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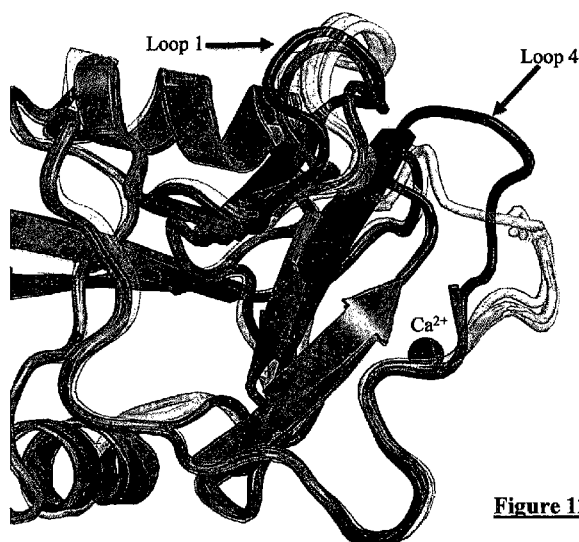


Figure 12

(57) **Abstract:** The invention relates to antibodies, and in particular to IgE antibodies. The invention is primarily concerned with variant Fc regions of IgE antibodies and their use in the creation of novel engineered or modified IgE antibodies. The invention extends to methods for modifying antibodies and to pharmaceutical compositions comprising such antibodies. The invention also covers uses of the modified antibodies in a wide range of therapeutic applications.



VARIANT IGE FC REGIONS

The present invention relates to antibodies, and in particular to immunoglobulin E (IgE) antibodies. The invention is primarily concerned with variant Fc regions of IgE antibodies and their use in the creation of novel engineered or modified IgE antibodies. The invention extends to methods for modifying antibodies and to pharmaceutical compositions comprising such antibodies. The invention also covers uses of the modified antibodies in a wide range of therapeutic applications.

Immunoglobulin E (IgE) antibodies play a key role in the mechanisms of allergic disease, not only recognizing allergens through their Fab regions, but also interacting *via* their Fc regions with two very different cell surface receptors. FcεRI, the receptor found on mast cells and basophils, binds IgE with high affinity ($K_A = 10^{10}$ - 10^{11} M⁻¹) and is responsible for allergic sensitization and the immediate (Type I) hypersensitivity reaction in which minute amounts of allergen cross-link receptor-bound IgE and trigger cell degranulation. The IgE-binding α-chain of FcεRI consists of two extracellular Ig-like domains (sFcεRIα).

In contrast, FcεRII (also known as CD23), expressed on B cells, consists of three C-type lectin “head” domains connected to the membrane by a trimeric α-helical coiled-coil “stalk”. A single head domain binds to IgE-Fc with lower affinity ($K_A = 10^5$ - 10^6 M⁻¹) than FcεRI, although avidity of the trimer can substantially enhance this interaction. Membrane CD23 (mCD23) is cleaved from the cell surface by endogenous proteases such as ADAM10 to yield soluble trimeric and monomeric forms (sCD23), which have been implicated in both positive and negative feedback mechanisms for the regulation of IgE synthesis by B cells that have switched to IgE production. Both FcεRI and CD23 are also expressed on a range of antigen-presenting cells (APC) where they play similar roles in trapping IgE-allergen complexes and promoting the allergic response, but the functional interplay (i.e. cooperation or competition) between these two receptors in the context of APCs is not well understood.

CD23 expressed on B cells also has the potential to contribute to the clinically serious phenomenon of the spreading of allergic reactivity to unrelated allergens, through its ability to internalize IgE-allergen complexes irrespective of the allergen, in contrast to mIgE-mediated allergen-specific presentation through the B cell receptor. CD23 expressed on gastrointestinal epithelial cells also contributes to IgE-allergen

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transport across the gut epithelial barrier to trigger food allergenic reactions and similarly on respiratory tract epithelial cells to contribute to airway allergic inflammation. Understanding the IgE-CD23 interaction thus has implications for many aspects of allergic disease.

5

As described in the Examples, using x-ray crystallography, NMR spectroscopy and site-directed mutagenesis, the inventors have now accurately defined the CD23 binding site on the Fc region of IgE, and have clearly demonstrated that the binding site is located in the C ϵ 3 domain of IgE. Based on these results, the inventors have created a series of variant IgE Fc regions, which may be used in the creation of novel and clinically safer IgE-based antibody therapies.

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Therefore, in a first aspect of the invention, there is provided a variant IgE Fc region comprising at least one amino acid modification in the C ϵ 3 domain relative to a wild-type IgE Fc region, wherein the at least one amino acid in the wild-type IgE Fc region which is modified is part of a CD23 binding site, and wherein the variant IgE Fc region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc region.

15

Advantageously, and preferably, the inventors have found that the variant IgE Fc region of the first aspect exhibits reduced, and preferably ablated, binding to CD23 (Fc ϵ RII), but is capable of binding to Fc ϵ RI. Thus, the variant IgE Fc region may still bind to antigens and trigger mast cells through its interaction with Fc ϵ RI, but, because it cannot bind (or only poorly binds) to CD23, it avoids the problematic side effects that are commonly associated with CD23 binding, including epitope spreading. Therefore, the Fc region of the invention has considerable utility in IgE antibody engineering. The inventors have surprisingly shown that the CD23 and Fc ϵ RI binding sites occur on opposite ends of the C ϵ 3 domain of IgE, and that allosteric inhibition prohibits simultaneous binding of these two receptors, thereby preventing engagement and cross-linking of IgE bound to mast cells by soluble CD23.

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It will be appreciated that immunoglobulin E (IgE) is a class of antibodies found only in mammals. IgE is a dimeric antibody with four Ig-like domains (C ϵ 1- C ϵ 4). The ability to mediate cytotoxic and phagocytic effector functions are potent mechanisms by which antibodies destroy targeted cells.

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The Fc region links the recognition domain of antibodies to these effector functions through an interaction with Fc receptors and ligands. Manipulation of these effector functions by alteration of the Fc region has important implications in the treatment of numerous medical conditions, e.g. cancer, autoimmune disease and infectious diseases. The Fc region comprises the constant region of an antibody excluding the first constant region immunoglobulin domain. Wild type Fc region refers to the amino acid or nucleotide sequences that are found in nature, including allelic variations. Variant Fc region refers to an Fc sequence that differs from the wild type sequence by virtue of at least one amino acid modification. An Fc variant may only encompass an Fc region, or it may exist in the context of an antibody, Fc fusion, isolated Fc, Fc fragment, or other polypeptide that is substantially encoded by Fc. Fc variant may refer to the Fc polypeptide itself, compositions comprising the Fc variant polypeptide or the amino acid sequence.

The DNA sequence encoding wild-type human IgE-Fc is provided herein as SEQ ID No:1, as follows:

```
TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCTGCGACGGCGGC
GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGACT
ATCAACATCACCTGGCTGGAGGACGGGAGGTCATGGACGTGGACTTGCCACCGCCTCT
ACCACGCAGGAGGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCCCTCAGCCAGAAGCAC
TGGCTGTCAGACCGCACCTACACCTGCCAGGTACCTATCAAGGTCACACCTTGAGGAC
AGCACCAAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCACGATCACCTGTCTGGTGGTGACCTG
GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAAC
CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCAGCTTAACCGTCACGTCCACCCCTG
CCGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCAAGTGCAGGGTGACCCACCCC
CACCTGCCCCAGGGCCCTCATGCGGTCCACGACCAAGACCGGGCCCGCGTGTGCCCCG
GAAGTCTATGCGTTTTCGACGCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCCCTCGCC
TGCCTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
CAGTCCCGGACGCGCGGCACAGCAGCAGCGAGCCCGCAAGACCAAGGGCTCCGGCTTC
TTCGTCTTACCGCGCTGGAGGTGACCAAGGCGCAATGGGAGCAGAAAGATGAGTTCATC
TGCCGTGCAGTCCATGAGGCAGCGAGCCCTCACAGACCGTCCAGCGAGCGGTGTCTGTA
AATCCCG
```

[SEQ ID No:1]

The polypeptide sequence of wild-type human IgE-Fc is provided herein as SEQ ID No:2, as follows:

```
CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS 283
TTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP 343
SPFDLFIKSPITITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTL 403
PVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLA 463
CLIQNFMPEDISVQWLHNEVQLPDRHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI 523
CRVHEAASPSTVQRAVSVNP
```

[SEQ ID No:2]

Thus, preferably the variant IgE Fc region of the invention is derived from the wild-type human IgE-Fc comprising an amino acid sequence substantially as set out in SEQ ID No:2, or a functional variant or fragment thereof. Preferably, the variant IgE Fc region is derived from the wild-type human IgE-Fc which is encoded by a nucleic

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acid sequence substantially as set out in SEQ ID No:1, or a functional variant or fragment thereof.

The C ϵ 3 domain of IgE is defined as amino acid residues 329-441 of SEQ ID No:2.

5 Thus, the at least one amino acid which is modified may be any one of amino acid residues 329-441 of SEQ ID No:2.

As described in the Examples, and as illustrated in Figure 1B, the inventors are the first to demonstrate that the A-B helix of C ϵ 3 does not form part of the CD23 binding
10 site, and so it cannot be directly involved in CD23 binding. Therefore, preferably the at least one amino acid modification in the variant IgE Fc region is not in the A-B helix of the C ϵ 3 domain of IgE. It will be appreciated that the A-B helix of the C ϵ 3 domain of IgE is defined as amino acid residues 344-353 of SEQ ID No:2.

15 The inventors have observed that a number of amino acid residues from three discontinuous sequences in the Fc region of IgE form a contiguous surface, which together represent the binding site on the C ϵ 3 domain of IgE for the CD23 receptor. These three spaced apart sequences are located in the E-F helix, the C-D loop and the C-terminal region of the C ϵ 3 domain of IgE. Accordingly, it is preferred that the
20 variant IgE Fc region comprises at least one amino acid modification in the E-F helix of the C ϵ 3 domain of IgE, in the C-D loop of the C ϵ 3 domain of IgE and/or in the C-terminal region of the C ϵ 3 domain of IgE.

It will be appreciated that the E-F helix of the C ϵ 3 domain of IgE is defined as amino
25 acid residues 404-415 of SEQ ID No:2, the C-D loop of the C ϵ 3 domain of IgE is defined as amino acid residues 376-380 of SEQ ID No:2, and that the C-terminal region of the C ϵ 3 domain of IgE is defined as amino acid residues 433-441 of SEQ ID No:2. It will be appreciated that the numbering of amino acid residues given herein is equivalent to the wild-type sequence of human Ig Fc region as shown in SEQ ID
30 No:2.

Therefore, preferably the variant IgE Fc region comprises at least one amino acid modification at a position corresponding to wild-type IgE Fc region selected from the group consisting of: (i) amino acids 404-415 from the E-F helix of IgE; (ii) amino
35 acids 376-380 from the C-D loop of IgE; and (iii) residue 433-441 from the C-terminal region of IgE. Thus, the variant IgE region may comprise at least one amino

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acid modification at a position corresponding to wild-type IgE Fc region selected from the group consisting of: 376; 377; 378; 379; 380; 404; 405; 406; 407; 408; 409; 410; 411; 412; 413; 414; 415; 433; 434; 435; 436; 437; 438; 439; 440; and 441 of SEQ ID No:2.

5

The inventors have analysed the bound structure of the IgE/CD23 complex and used considerable inventive endeavour to determine the solvent-exposed IgE residues that make contact with CD23. Accordingly, the variant IgE region may comprise at least one amino acid modification at a position corresponding to wild-type IgE Fc region
10 selected from the group consisting of: 376; 378; 381; 408; 409; 411; 412; 435; 439; and 440.

15

The inventors observed that mutations of amino acid residues at positions 409, 412, 376, 380 and 435 displayed the largest effects on CD23 binding energetics. Therefore, preferably the variant IgE region comprises at least one amino acid modification at a position equivalent to 409, 412, 376, 380 and/or 435 of the wild-type human IgE Fc region.

20

The at least one modification may comprise an amino acid deletion or an insertion. However, preferably the at least one modification comprises an amino acid substitution. Preferably, the at least one modification may comprise a non-conservative amino acid change. For example, the charge (i.e. pH) of amino acid may be changed, or the residue may be converted from being hydrophilic so that it is hydrophobic, or vice versa. For example, a K residue may be substituted for a D, or an
25 E may be replaced with a R. Alternatively, a small amino acid may be replaced with a large amino acid (e.g., Y for A).

30

Alternatively, a large amino acid may be replaced with a small or constraining amino acid, for example alanine, glycine, proline or serine (e.g., F may be replaced with S). Substitution with alanine may be preferred, and the skilled person would readily appreciate the codons which encode alanine, and therefore how to modify the wild-type DNA sequence of SEQ ID No:1 to produce variant Fc regions of the invention. For example, in one embodiment the variant Fc region may comprise at least one amino acid modification selected from: D409A; E412A; R376A; K380A and K435A.

35

The variant human IgE Fc region may comprise more than one modification in the Cε3 domain of the Fc region equivalent to the human IgE Fc region of a wild-type

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antibody. For example, the variant human IgE Fc region may comprise at least two, three, four or five modifications in the Cε3 domain, as described herein. Thus, the variant Fc region may comprise a double or treble mutant, and so on. The variant Fc region may comprise a modification at two, three, four, five or more of the amino acids at positions equivalent to 376; 377; 378; 379; 380; 404; 405; 406; 407; 408; 409; 410; 411; 412; 413; 414; 415; 433; 434; 435; 436; 437; 438; 439; 440; and 441 of wild-type human IgE-Fc. The variant Fc region may comprise a modification at each of the amino acids at positions equivalent to 409, 412, 376, 380 and/or 435 of wild-type human IgE-Fc.

In one embodiment, the polypeptide sequence of the variant IgE Fc region (denoted as D409A) is provided herein as SEQ ID No:3, as follows:

```

CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS 283
TTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP 343
SPFDLFIKRSPTITCLVVDLAPSKGTVNLWTSRASGKPVNHSTRKEEKQRNGTLTVTSTL 403
PVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLA 463
CLIQNFMPEDISVQWLHNEVQLPDARHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI 523
CRAVHEAASPSQTVQRAVSVNP

```

[SEQ ID No:3]

In another embodiment, the polypeptide sequence of the variant IgE Fc region (E412A) is provided herein as SEQ ID No:4, as follows:

```

CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS 283
TTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP 343
SPFDLFIKRSPTITCLVVDLAPSKGTVNLWTSRASGKPVNHSTRKEEKQRNGTLTVTSTL 403
PVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLA 463
CLIQNFMPEDISVQWLHNEVQLPDARHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI 523
CRAVHEAASPSQTVQRAVSVNP

```

[SEQ ID No:4]

In another embodiment, the polypeptide sequence of the variant IgE Fc region (R376A) is provided herein as SEQ ID No:5, as follows:

```

CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS 283
TTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP 343
SPFDLFIKRSPTITCLVVDLAPSKGTVNLWTSRASGKPVNHSTRKEEKQRNGTLTVTSTL 403
PVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLA 463
CLIQNFMPEDISVQWLHNEVQLPDARHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI 523
CRAVHEAASPSQTVQRAVSVNP

```

[SEQ ID No:5]

In another embodiment, the polypeptide sequence of the variant IgE Fc region (K380A) is provided herein as SEQ ID No:6, as follows:

```

CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS 283
TTQEGELASTQSELTLSQKHWLSDRTYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP 343
SPFDLFIKRSPTITCLVVDLAPSKGTVNLWTSRASGKPVNHSTRKEEKQRNGTLTVTSTL 403
PVGTRDWIEGETYQCRVTHPHLPRALMRSTTKTSGPRAAPEVYAFATPEWPGSRDKRTLA 463
CLIQNFMPEDISVQWLHNEVQLPDARHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI 523
CRAVHEAASPSQTVQRAVSVNP

```

[SEQ ID No:6]

In another embodiment, the polypeptide sequence of the variant IgE Fc region (K435A) is provided herein as SEQ ID No:7, as follows:

```

5      CSRDFTPPTVKILQSSCDGGGHFPPTIQLLCLVSGYTPGTINITWLEDGQVMDVDLSTAS  283
      TTQEGELASTQSELTLSQKHWSLDRITYTCQVTYQGHTFEDSTKKCADSNPRGVSAYLSRP  343
      SPFDLFIKRSPTITCLVVDLAPSKGTVNLTWSRASGKPVNHSTRKEEKQRNGTLTVTSTL  403
      PVGTRDWIEGETYQCRVTHPLPRALMRSTTATSGPRAAPEVYAFATPEWPGSRDKRTLA  463
10     CLIQNFMPEDISVQWLHNEVQLPDARHSTTQPRKTKGSGFFVFSRLEVTRAWEQKDEFI  523
      CRVHEEAASPSQTVQRAVSVNP

```

[SEQ ID No:7]

Thus, a preferred variant IgE Fc region of the invention comprises an amino acid sequence substantially as set out in any one of SEQ ID No: 3-7, or a functional variant or fragment thereof.

The IgE Fc variants disclosed herein exhibit reduced binding affinity to the CD23 receptor compared to that of the wild-type or parent Fc region. The term "reduced affinity" compared to the wild-type Fc region can mean that the Fc variant binds to CD23 with a lower equilibrium constant of association (K_A or K_a) or higher equilibrium constant of dissociation (K_D or K_d) than the wild-type Fc region when the amounts of variant and wild type region in the binding assay that is used are essentially the same. For example, the Fc variant with reduced binding affinity for CD23 may display from about a 5-fold to about 10,000-fold reduction in CD23 binding affinity compared to the wild type Fc region, where CD23 binding affinity is determined, for example, by the binding methods disclosed herein.

Reduced affinity can also be defined relative to an absolute level of affinity. As shown in Table 1, the preferred variants exhibit surprisingly high K_D values. For example, the equilibrium constant of dissociation (K_D or K_d) of the variant IgE Fc region for CD23 may be at least 5 μ M, 10 μ M, 25 μ M or more.

Preferably, the variant IgE Fc region of the first aspect is capable of binding to Fc ϵ RI. Preferably, the binding affinity of the variant Fc region for Fc ϵ RI is at least the same as that of the wild-type Fc region. For example, the variant IgE Fc region is capable of binding to Fc ϵ RI with a K_A value of at least 10⁷ M⁻¹, 10⁷ M⁻¹, 10⁸ M⁻¹, 10⁹ M⁻¹, or at least 10¹⁰ M⁻¹.

In a second aspect, there is provided a nucleic acid encoding the variant IgE Fc region according to the first aspect.

In one embodiment, the DNA sequence encoding the variant IgE Fc region (D409A) is provided herein as SEQ ID No:8, as follows:

5 TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGC
GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGGACT
ATCAACATCACTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCT
10 ACCACGCAGGAGGGTGAGCTGGCTCCACACAAAGCGAGCTACCCCTCAGCCAGAAGCAC
TGGCTGTGACACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGAC
AGCACCAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCCACGATCACCTGTCTGGTGGTGACCTG
GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAAC
CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCCTG
15 CCGGTGGGCACCCGAGCTTGGATCGAGGGGAGACCTACCAAGTGCAGGGTGACCCACCCC
CACCTGCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGTGCCCCG
GAAGTCTATGCGTTTTCGACGCGCGGAGTGGCCGGGGAGCCGGGACAAAGCGCACCCCTCGCC
TGCTGATCCAGAACTTCATGCGCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
CAGCTCCCGGACGCCCGGCACAGCACGACGACGCCCCGCAAGACCAAGGGCTCCGGCTTC
TTCGTCTTACGCCGCTGGAGGTGACCAAGGGCCGAATGGGAGCAGAAAGATGAGTTTCATC
20 TGCCGTGCAGTCCATGAGGCAGCGAGCCCCCTACAGACCGTCCAGCGAGCGGTGTCTGTA
AATCCCG

[SEQ ID No:8]

In another embodiment, the DNA sequence encoding the variant IgE Fc region (E412A) is provided herein as SEQ ID No:9, as follows:

25 TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGC
GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGGACT
ATCAACATCACTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCT
30 ACCACGCAGGAGGGTGAGCTGGCTCCACACAAAGCGAGCTACCCCTCAGCCAGAAGCAC
TGGCTGTGACACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGAC
AGCACCAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCCACGATCACCTGTCTGGTGGTGACCTG
GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAAC
CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCCTG
35 CCGGTGGGCACCCGAGACTGGATCGAGGGGAGACCTACCAAGTGCAGGGTGACCCACCCC
CACCTGCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGTGCCCCG
GAAGTCTATGCGTTTTCGACGCGCGGAGTGGCCGGGGAGCCGGGACAAAGCGCACCCCTCGCC
TGCTGATCCAGAACTTCATGCGCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
CAGCTCCCGGACGCCCGGCACAGCACGACGACGCCCCGCAAGACCAAGGGCTCCGGCTTC
40 TTCGTCTTACGCCGCTGGAGGTGACCAAGGGCCGAATGGGAGCAGAAAGATGAGTTTCATC
TGCCGTGCAGTCCATGAGGCAGCGAGCCCCCTACAGACCGTCCAGCGAGCGGTGTCTGTA
AATCCCG

[SEQ ID No:9]

45

In another embodiment, the DNA sequence encoding the variant IgE Fc region (R376A) is provided herein as SEQ ID No:10, as follows:

50 TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGC
GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGGACT
ATCAACATCACTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCT
ACCACGCAGGAGGGTGAGCTGGCTCCACACAAAGCGAGCTACCCCTCAGCCAGAAGCAC
TGGCTGTGACACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGAC
AGCACCAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
55 AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCCACGATCACCTGTCTGGTGGTGACCTG
GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAAC
CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCACGTTAACCGTCACGTCCACCCCTG
CCGGTGGGCACCCGAGACTGGATCGAGGGGAGACCTACCAAGTGCAGGGTGACCCACCCC
CACCTGCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGTGCCCCG
GAAGTCTATGCGTTTTCGACGCGCGGAGTGGCCGGGGAGCCGGGACAAAGCGCACCCCTCGCC
60 TGCTGATCCAGAACTTCATGCGCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
CAGCTCCCGGACGCCCGGCACAGCACGACGACGCCCCGCAAGACCAAGGGCTCCGGCTTC
TTCGTCTTACGCCGCTGGAGGTGACCAAGGGCCGAATGGGAGCAGAAAGATGAGTTTCATC
TGCCGTGCAGTCCATGAGGCAGCGAGCCCCCTACAGACCGTCCAGCGAGCGGTGTCTGTA
65 AATCCCG

[SEQ ID No:10]

- 9 -

In another embodiment, the DNA sequence encoding the variant IgE Fc region (K380A) is provided herein as SEQ ID No:11, as follows:

```

5      TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGC
      GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGACT
      ATCAACATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCT
      ACCACGCAGGAGGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCTCAGCCAGAAGCAC
      TGGCTGTCAGACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGAC
      AGCACCAAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
      AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCCACGATCACCTGTCTGGTGGTGGACCTG
      10    GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGACCTGTGAAC
      CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCAGGTTAACCCTCACGTCCACCTTG
      CCGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCACTGCAGGGTGACCCACCCC
      CACCTGCCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGTGCCCGG
      15    GAAGTCTATGCGTTTTCGACGCGCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCTCGCC
      TGCCTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
      CAGTCCCGGACGCGCCGACAGCAGCAGCAGCCCGCAAGACCAAGGGCTCCGGCTTC
      TTCGTCTTCAGCCGCTGGAGGTGACCAGGGCCGAATGGGAGCAGAAAGATGAGTTTCATC
      TGCCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCCAGCGAGCGGTGTCTGTA
      AATCCCG
20

```

[SEQ ID No:11]

In another embodiment, the DNA sequence encoding the variant IgE Fc region (K435A) is provided herein as SEQ ID No:12, as follows:

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25    TGCTCCAGGGACTTCACCCCGCCACCGTGAAGATCTTACAGTCGTCCTGCGACGGCGGC
      GGGCACTTCCCCCGACCATCCAGCTCCTGTGCCTCGTCTCTGGGTACACCCAGGGACT
      ATCAACATCACCTGGCTGGAGGACGGGCAGGTCATGGACGTGGACTTGTCCACCGCCTCT
      ACCACGCAGGAGGGTGAGCTGGCCTCCACACAAAGCGAGCTCACCTCAGCCAGAAGCAC
      TGGCTGTCAGACCGCACCTACACCTGCCAGGTCACCTATCAAGGTCACACCTTTGAGGAC
      30    AGCACCAAGAAGTGTGCAGATTCCAACCCGAGAGGGGTGAGCGCCTACCTAAGCCGGCCC
      AGCCCGTTTCGACCTGTTTCATCCGCAAGTCGCCCACGATCACCTGTCTGGTGGTGGACCTG
      GCACCCAGCAAGGGGACCGTGAACCTGACCTGGTCCCGGGCCAGTGGGAAGCCTGTGAAC
      CACTCCACCAGAAAGGAGGAGAAGCAGCGCAATGGCAGGTTAACCCTCACGTCCACCTTG
      CCGGTGGGCACCCGAGACTGGATCGAGGGGGAGACCTACCACTGCAGGGTGACCCACCCC
      35    CACCTGCCCAGGGCCCTCATGCGGTCCACGACCAAGACCAGCGGCCCGCGTGTGCCCGG
      GAAGTCTATGCGTTTTCGACGCGCGGAGTGGCCGGGGAGCCGGGACAAGCGCACCTCGCC
      TGCCTGATCCAGAACTTCATGCCTGAGGACATCTCGGTGCAGTGGCTGCACAACGAGGTG
      CAGTCCCGGACGCGCCGACAGCAGCAGCAGCCCGCAAGACCAAGGGCTCCGGCTTC
      40    TTCGTCTTCAGCCGCTGGAGGTGACCAGGGCCGAATGGGAGCAGAAAGATGAGTTTCATC
      TGCCGTGCAGTCCATGAGGCAGCGAGCCCCTCACAGACCGTCCAGCGAGCGGTGTCTGTA
      AATCCCG

```

[SEQ ID No:12]

45 Thus, the nucleic acid according to the second aspect may comprise a nucleotide sequence substantially as set out in any one of SEQ ID No: 8-12, or a functional variant or fragment thereof. For each of the nucleic acid sequences represented by SEQ ID No: 8-12, the codon which is shown in bold and underlined represents a modification which would encode alanine. However, it will be appreciated that

50 alanine may be encoded by GCT, GCC, GCA and GCG and so any of these codons would be suitable as a modification to create the same amino acid mutation. Similarly, the codons for glycine (GGT, GGC, GGA and GGG), proline (CCT, CCC, CCA, CCG) and serine (TCT, TCC, TCA, TCG, AGT and AGC) will be known, and could be used to modify wild type sequence.

55

The nucleic acid may be an isolated or purified nucleic acid sequence. The nucleic acid sequence may be a DNA sequence. Also described herein are methods for

- 10 -

producing the variant IgE Fc region of the first aspect using the nucleic acids of the second aspect. Thus, the nucleic acid sequence may be incorporated into a genetic construct for cloning purposes.

- 5 In a third aspect, there is provided a genetic construct comprising the nucleic acid of the second aspect.

Genetic constructs of the invention may be in the form of an expression cassette, which may be suitable for expression of the encoded polypeptide (i.e. the variant IgE
10 Fc region) in a host cell. The genetic construct may be introduced in to a host cell without it being incorporated in a vector. For instance, the genetic construct, which may be a nucleic acid molecule, may be incorporated within a liposome or a virus particle. Alternatively, a purified nucleic acid molecule (e.g. histone-free DNA, or naked DNA) may be inserted directly into a host cell by suitable means, e.g. direct
15 endocytotic uptake. The genetic construct may be introduced directly in to cells of a host subject (e.g. a bacterial or eukaryotic cell) by transfection, infection, electroporation, microinjection, cell fusion, protoplast fusion or ballistic bombardment. Alternatively, genetic constructs of the invention may be introduced directly into a host cell using a particle gun. Alternatively, the genetic construct may
20 be harboured within a recombinant vector, for expression in a suitable host cell.

Therefore, in a fourth aspect, there is provided a recombinant vector comprising the genetic construct according to the third aspect.

- 25 The recombinant vector may be a plasmid, cosmid or phage. Such recombinant vectors are useful for transforming host cells with the genetic construct of the third aspect, and for replicating the expression cassette therein. The skilled technician will appreciate that genetic constructs of the invention may be combined with many types of backbone vector for expression purposes. Recombinant vectors may include a
30 variety of other functional elements including a suitable promoter to initiate gene expression. For instance, the recombinant vector may be designed such that it autonomously replicates in the cytosol of the host cell. In this case, elements which induce or regulate DNA replication may be required in the recombinant vector. Alternatively, the recombinant vector may be designed such that it integrates into the
35 genome of a host cell. In this case, DNA sequences which favour targeted integration (e.g. by homologous recombination) are envisaged.

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The recombinant vector may also comprise DNA coding for a gene that may be used as a selectable marker in the cloning process, i.e. to enable selection of cells that have been transfected or transformed, and to enable the selection of cells harbouring vectors incorporating heterologous DNA. Alternatively, the selectable marker gene
5 may be in a different vector to be used simultaneously with vector containing the gene of interest. The vector may also comprise DNA involved with regulating expression of the coding sequence, or for targeting the expressed polypeptide to a certain part of the host cell.

10 In a fifth aspect, there is provided a host cell comprising the genetic construct according to the third aspect, or the recombinant vector according to the fourth aspect.

The host cell may be a bacterial cell. The host cell may be an animal cell. The host
15 cell may be a mammalian cell, for example a mouse or rat cell. It is preferred that the host cell is not a human cell. The host cell may be transformed with genetic constructs or vectors according to the invention, using known techniques. Suitable means for introducing the genetic construct into the host cell will depend on the type of cell.

20 Thus, according to a sixth aspect, there is provided a method of preparing a variant IgE Fc region, the method comprising- (i) culturing at least one cell according to the fifth aspect under conditions suitable for the expression of a variant IgE Fc region; and (ii) isolating the variant IgE Fc region.

25 The inventors believe that knowledge of the sequences of IgE Fc region which form the binding site for the CD23 receptor can be harnessed in a method for reducing the binding affinity of any existing IgE antibody or in the production of novel IgE antibodies, in order to reduce or avoid the risks of patients suffering from any CD23-
30 mediated side effects that may be associated with an IgE antibody therapy.

Hence, in a seventh aspect, there is provided a method of producing a variant IgE Fc region which exhibits a reduced binding affinity for CD23, the method comprising modifying at least one amino acid in the C ϵ 3 domain of a wild-type IgE Fc region,
35 wherein the at least one amino acid in the wild-type IgE Fc region which is modified

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is part of the CD23 binding site, such that the variant IgE Fc region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc region.

Preferably, the variant IgE Fc region produced by the method is incapable of binding
5 to CD23 (FcεRII), but is capable of binding to FcεRI.

Preferably, the method does not comprise modifying an amino acid in the A-B helix of the Cε3 domain of IgE. Preferably, the method comprises modifying at least one amino acid in the E-F helix, in the C-D loop and/or in the C-terminal region of the
10 Cε3 domain of IgE. Preferably, the method comprises modifying at least one amino acid at a position corresponding to wild-type IgE Fc region selected from the group consisting of: (i) amino acids 404-415 from the E-F helix of IgE; (ii) amino acids 376-380 from the C-D loop of IgE; and (iii) residue 433-441 from the C-terminal region of IgE. The method may comprise modifying at least one amino acid at a position
15 corresponding to wild-type IgE Fc region selected from the group consisting of: 376; 377; 378; 379; 380; 404; 405; 406; 407; 408; 409; 410; 411; 412; 413; 414; 415; 433; 434; 435; 436; 437; 438; 439; 440; and 441 of SEQ ID No:2. Preferably, the method comprises modifying at least one amino acid at a position equivalent to 409, 412, 376, 380 and/or 435 of the wild-type human IgE Fc region. The method may comprise
20 modifying at least two, three, four, five or more amino acids in the Cε3 domain relative to a wild-type IgE Fc region.

The wild-type IgE Fc region which is modified in the method may be part of a therapeutically active IgE antibody. Examples of such an IgE antibody which may be
25 modified include IgE antibodies directed against tumour antigens, as described by Karagiannis *et al.* (Karagiannis, 2011, *Cancer Immunol Immunotherapy*; PMID 22139135). Thus, the method may be used to modify the IgE antibody such that it cannot bind to CD23, thereby avoiding the risk of CD23-mediated side effects.

Alternatively, the method may comprise initially creating the variant IgE Fc region and then fusing it to a functional fragment of an IgE antibody to produce an Fc fusion, for example a chimera. The functional fragment may comprise variable regions exhibiting immunospecificity for a target epitope. The functional fragment may be selected from a group consisting of VH, VL, Fd, Fv, Fab, Fab', scFv and
35 F(ab')₂.

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Based on the foregoing, it will be appreciated that the variant IgE Fc region of the first aspect or the method of the seventh aspect can be used to form antibody or a functional fragment thereof.

5 Thus, in an eighth aspect of the invention, there is provided an antibody or a functional fragment thereof comprising the variant IgE Fc region of the first aspect.

Preferably, the antibody or functional fragment thereof is incapable of binding to CD23 (FcεRII), or exhibits a reduced binding affinity thereto, but is capable of
10 binding to FcεRI.

The invention extends both to whole antibodies (i.e. immunoglobulins) with immunospecificity for a certain target epitope, as well as to functional fragments thereof. Such fragments retain at least one antigen binding region of a corresponding
15 full-length antibody as well as the variant IgE Fc region of the invention. The antibody or functional fragment thereof may comprise a monoclonal or polyclonal antibody or functional fragment thereof.

The antibody or functional fragment may be monovalent, divalent or polyvalent.
20 Monovalent antibodies are dimers (HL) comprising a heavy (H) chain associated by a disulfide bridge with a light chain (L). Divalent antibodies are tetramer (H₂L₂) comprising two dimers associated by at least one disulfide bridge. Polyvalent antibodies may also be produced, for example by linking multiple dimers. The basic structure of an antibody molecule consists of two identical light chains and two
25 identical heavy chains which associate non-covalently and can be linked by disulphide bonds. Each heavy and light chain contains an amino-terminal variable region of about 110 amino acids, and constant sequences in the remainder of the chain. The variable region includes several hypervariable regions, or complementarity determining regions (CDRs), that form the antigen-binding site of
30 the antibody molecule and determine its specificity for the antigen in question. On either side of the CDRs of the heavy and light chains is a framework region, a relatively conserved sequence of amino acids that anchors and orients the CDRs.

The constant region consists of one of five heavy chain sequences (μ, γ, ζ, α, or ε) and
35 one of two light chain sequences (κ or λ). The heavy chain constant region sequences determine the isotype of the antibody and the effector functions of the molecule.

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Preferably, the antibody or functional fragment thereof is an IgE.

The antibody or fragment thereof may be a human antibody. As used herein, the term “human antibody” can mean an antibody, such as a monoclonal antibody, which
5 comprises substantially the same heavy and light chain CDR amino acid sequences as found in a particular human antibody exhibiting immunospecificity for certain epitope. An amino acid sequence, which is substantially the same as a heavy or light chain CDR, exhibits a considerable amount of sequence identity when compared to a reference sequence. Such identity is definitively known or recognizable as
10 representing the amino acid sequence of the particular human antibody.

Substantially the same heavy and light chain CDR amino acid sequence can have, for example, minor modifications or conservative substitutions of amino acids. Such a human antibody maintains its function of selectively binding to an epitope.

15 The term “human monoclonal antibody” can include a monoclonal antibody with substantially human CDR amino acid sequences produced, for example by recombinant methods such as production by a phage library, by lymphocytes or by hybridoma cells.

20 The term “humanised antibody” can mean an antibody from a non-human species (e.g. mouse) whose protein sequences have been modified to increase their similarity to antibodies produced naturally in humans.

The antibody may be a recombinant antibody. The term “recombinant human
25 antibody” can include a human antibody produced using recombinant DNA technology.

The term “antigen binding region” can mean a region of the antibody having specific binding affinity for its target antigen. The binding region may be a hypervariable
30 CDR or a functional portion thereof. The term “functional portion” of a CDR can mean a sequence within the CDR which shows specific affinity for the target antigen. The functional portion of a CDR may comprise a ligand which specifically binds to its epitope.

35 The term “CDR” can mean a hypervariable region in the heavy and light variable chains. There may be one, two, three or more CDRs in each of the heavy and light chains of the antibody. Normally, there are at least three CDRs on each chain which,

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when configured together, form the antigen-binding site, i.e. the three-dimensional combining site with which the antigen binds or specifically reacts. It has however been postulated that there may be four CDRs in the heavy chains of some antibodies. The definition of CDR also includes overlapping or subsets of amino acid residues
5 when compared against each other. The exact residue numbers which encompass a particular CDR or a functional portion thereof, will vary depending on the sequence and size of the CDR. Those skilled in the art can routinely determine which residues comprise a particular CDR given the variable region amino acid sequence of the antibody.

10

The term "functional fragment" of an antibody can mean a portion of the antibody which retains a functional activity. A functional activity can be, for example antigen binding activity or specificity. A functional activity can also be, for example, an effector function provided by an antibody constant region. The term "functional
15 fragment" is also intended to include, for example, fragments produced by protease digestion or reduction of a human monoclonal antibody and by recombinant DNA methods known to those skilled in the art. Human monoclonal antibody functional fragments include, for example individual heavy or light chains and fragments thereof, such as VL, VH and Fd; monovalent fragments, such as Fv, Fab, and Fab' ;
20 bivalent fragments such as F(ab')₂; single chain Fv (scFv); and Fc fragments.

25

The term "VL fragment" can mean a fragment of the light chain of a human monoclonal antibody which includes all or part of the light chain variable region, including the CDRs. A VL fragment can further include light chain constant region
sequences.

30

The term "VH fragment" can mean a fragment of the heavy chain of a human monoclonal antibody which includes all or part of the heavy chain variable region, including the CDRs.

35

The term "Fd fragment" can mean the light chain variable and constant regions coupled to the heavy chain variable and constant regions, i.e. VL CL and VH CH-1.

The term "Fv fragment" can mean a monovalent antigen-binding fragment of a human monoclonal antibody, including all or part of the variable regions of the heavy and light chains, and absent of the constant regions of the heavy and light chains. The variable regions of the heavy and light chains include, for example, the CDRs. For

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example, an Fv fragment includes all or part of the amino terminal variable region of about 110 amino acids of both the heavy and light chains.

5 The term “Fab fragment” means a monovalent antigen-binding fragment of a human monoclonal antibody that is larger than an Fv fragment. For example, a Fab fragment includes the variable regions, and all or part of the first constant domain of the heavy and light chains. Thus, a Fab fragment additionally includes, for example, amino acid residues from about 110 to about 220 of the heavy and light chains.

10 The term “Fab' fragment” can means a monovalent antigen-binding fragment of a human monoclonal antibody that is larger than a Fab fragment. For example, a Fab' fragment includes all of the light chain, all of the variable region of the heavy chain, and all or part of the first and second constant domains of the heavy chain. For example, a Fab' fragment can additionally include some or all of amino acid residues
15 220 to 330 of the heavy chain.

The term “F(ab')₂ fragment” can mean a bivalent antigen-binding fragment of a human monoclonal antibody. An F(ab')₂ fragment includes, for example, all or part of the variable regions of two heavy chains-and two light chains, and can further include
20 all or part of the first constant domains of two heavy chains and two light chains.

The term “single chain Fv (scFv)” can mean a fusion of the variable regions of the heavy (VH) and light chains (VL) connected with a short linker peptide.

25 One skilled in the art knows that the exact boundaries of a fragment of a human monoclonal antibody are not important, so long as the fragment maintains a functional activity. Using well-known recombinant methods, one skilled in the art can engineer a polynucleotide sequence to express a functional fragment with any endpoints desired for a particular application. A functional fragment of the antibody
30 may comprise fragments with substantially the same heavy and light chain variable regions as the human antibody.

The functional fragment may include fragments wherein at least one of the binding region sequences has substantially the same amino acid sequence as the binding
35 region sequences of the antibody. The functional fragment may comprise any of the fragments selected from a group consisting of VH, VL, Fd, Fv, Fab, Fab', scFv, F (ab')₂ and Fc fragment. Thus, in some embodiment, the invention comprises a functional

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fragment of an antibody (e.g. Fv, Fab, and Fab', bivalent fragments such as F(ab')₂, single chain Fv (scFv), fused to the Fc region of the first aspect.

The functional fragment may comprise any one of the antigen binding region
5 sequences of the VL, any one of the antigen binding region sequences of the VH, or a combination of VL and VH antigen binding regions of a human antibody. The appropriate number and combination of VH and VL antigen binding region sequences may be determined by those skilled in the art depending on the desired affinity and specificity and the intended use of the functional fragment. Functional
10 fragments of antibodies may be readily produced and isolated using methods well known to those skilled in the art. Such methods include, for example, proteolytic methods, recombinant methods and chemical synthesis. Proteolytic methods for the isolation of functional fragments comprise using human antibodies as a starting material. Enzymes suitable for proteolysis of human immunoglobulins may include,
15 for example, papain, and pepsin. The appropriate enzyme may be readily chosen by one skilled in the art, depending on, for example, whether monovalent or bivalent fragments are required. For example, papain cleavage results in two monovalent Fab' fragments that bind antigen and an Fc fragment. Pepsin cleavage, for example, results in a bivalent F (ab') fragment. An F (ab')₂ fragment of the invention may be
20 further reduced using, for example, DTT or 2-mercaptoethanol to produce two monovalent Fab' fragments.

Functional fragments produced by proteolysis may be purified by affinity and column chromatographic procedures. For example, undigested antibodies and Fc fragments
25 may be removed by binding to protein A. Additionally, functional fragments may be purified by virtue of their charge and size, using, for example, ion exchange and gel filtration chromatography. Such methods are well known to those skilled in the art.

The human antibody or functional fragment thereof may be produced by
30 recombinant methodology. Preferably, one initially isolates a polynucleotide encoding desired regions of the antibody heavy and light chains. Such regions may include, for example, all or part of the variable region of the heavy and light chains. Preferably, such regions can particularly include the antigen binding regions of the heavy and light chains, preferably the antigen binding sites, most preferably, the
35 CDRs.

The polynucleotide encoding the human antibody or functional fragment of the

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invention may be produced using methods known to those skilled in the art. The polynucleotide encoding the antibody or a functional fragment thereof may be directly synthesized by methods of oligonucleotide synthesis known in the art. Alternatively, smaller fragments may be synthesized and joined to form a larger
5 functional fragment using recombinant methods known in the art.

As used herein, the term “immunospecificity” can mean the binding region is capable of immunoreacting with a certain target epitope, by specifically binding therewith.

10 The term “immunoreact” can mean the binding region is capable of eliciting an immune response upon binding with an epitope.

The term “epitope” can mean any region of an antigen with ability to elicit, and combine with, a binding region of the antibody or fragment thereof.

15

Since IgE antibodies play a central role in the initiation and regulation of allergic disorders, clinical applications of the variant IgE Fc region of the first aspect, and of the antibodies (i.e. modified antibodies) of the eighth aspect, include their use in treating a wide range of medical conditions (including cancer, autoimmune disease or
20 infectious disease), while simultaneously avoiding the deleterious allergic side effects that are often observed in patients who are treated with IgE antibody therapies. In addition, they may also have utility in diagnosis.

25

Accordingly, in a ninth aspect of the invention, there is provided a variant IgE Fc region according to the first aspect, or an antibody or a functional fragment thereof according to the eighth aspect, for use in therapy or diagnosis.

30

In a tenth aspect, there is provided provided a variant IgE Fc region according to the first aspect, or an antibody or a functional fragment thereof according to the eighth aspect, for use in treating, preventing or ameliorating a CD23-mediated side effect.

35

In an eleventh aspect, there is provided a method for treating, preventing or ameliorating a CD23-mediated side effect in a subject, the method comprising administering, to a subject in need of such a treatment, a therapeutically effective amount of a variant IgE Fc region according to the first aspect, or an antibody or a functional fragment thereof according to the eighth aspect.

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Preferably, the variant IgE Fc region, or the antibody or a functional fragment thereof is used for preventing a CD23-mediated side effect. The CD23-mediated side effect, which is prevented or avoided, may be caused by IgE antibody treatment or therapy, of which there are many types. The CD23-mediated side effect which is prevented is one which may otherwise be caused during the treatment of cancer, autoimmune disease or infectious disease with a therapeutic IgE antibody. Thus, the antibody or fragment thereof may be a therapeutic antibody.

A CD23-mediated side effect which may be prevented may include CD23-dependent epitope spreading, which will be known to the skilled person. Epitope spreading is the process by which an antibody response to one epitope on an antigen leads to the production of antibodies specific for other epitopes on the same antigen, or for epitopes on entirely unrelated antigens. This results from the internalisation of whole antigen and subsequent display of a range of peptides derived from that antigen, leading to the generation of T cells with different epitope specificities. Simultaneous processing of two unrelated antigens by an antigen-presenting cell can lead to the production of antibodies directed against both antigens (Gould and Sutton, 2008, *Nature Reviews Immunology*, 8, 205-217).

It will be appreciated that antibodies, fragments, peptides and nucleic acids according to the invention (collectively referred to herein as “agents”) may be used in a monotherapy (e.g. the use of an antibody or fragment thereof alone), for treating, ameliorating or preventing a CD23-mediated side effect. Alternatively, agents according to the invention may be used as an adjunct to, or in combination with, known therapies for treating, ameliorating, or preventing CD23-mediated side effects.

The agents according to the invention may be combined in compositions having a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micellar solution, transdermal patch, liposome suspension or any other suitable form that may be administered to a person or animal in need of treatment. It will be appreciated that the vehicle of medicaments according to the invention should be one which is well-tolerated by the subject to whom it is given, and preferably enables delivery of the agents across the blood-brain barrier.

- 20 -

Medicaments comprising agents of the invention may be used in a number of ways. For instance, oral administration may be required, in which case the agents may be contained within a composition that may, for example, be ingested orally in the form of a tablet, capsule or liquid. Compositions comprising agents and medicaments of the invention may be administered by inhalation (e.g. intranasally). Compositions may also be formulated for topical use. For instance, creams or ointments may be applied to the skin.

Agents and medicaments according to the invention may also be incorporated within a slow- or delayed-release device. Such devices may, for example, be inserted on or under the skin, and the medicament may be released over weeks or even months. The device may be located at least adjacent the treatment site. Such devices may be particularly advantageous when long-term treatment with agents used according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

In a preferred embodiment, agents and medicaments according to the invention may be administered to a subject by injection into the blood stream or directly into a site requiring treatment. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion), or intradermal (bolus or infusion).

It will be appreciated that the amount of the antibodies, fragments, peptides and nucleic acids (i.e. agent) that is required is determined by its biological activity and bioavailability, which in turn depends on the mode of administration, the physiochemical properties of the agent, and whether it is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the half-life of the agent within the subject being treated. Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular agent in use, the strength of the pharmaceutical composition, the mode of administration, and the advancement of the disease. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

Generally, a daily dose of between 0.001µg/kg of body weight and 10mg/kg of body weight of agent according to the invention may be used for treating, ameliorating, or preventing a CD23-mediated side effect. More typically, the daily dose of agent is

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between 0.01µg/kg of body weight and 1mg/kg of body weight, more preferably between 0.1µg/kg and 100µg/kg body weight, and most preferably between approximately 0.1µg/kg and 10µg/kg body weight.

- 5 The agent may be administered before, during or after onset of the side effect. Daily doses may be given as a single administration (e.g. a single daily injection). Alternatively, the agent may require administration twice or more times during a day. As an example, agents may be administered as two (or more depending upon the severity of the condition being treated) daily doses of between 0.07 µg and 700 mg
- 10 (i.e. assuming a body weight of 70 kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3- or 4-hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses of agents according to the invention to a patient without the need to administer repeated doses. Known procedures, such as those conventionally
- 15 employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to form specific formulations of the agents according to the invention and precise therapeutic regimes (such as daily doses of the agents and the frequency of administration).
- 20 In a twelfth aspect of the invention, there is provided a pharmaceutical composition comprising a variant IgE Fc region according to the first aspect, or an antibody or fragment thereof according to the eighth aspect; and optionally a pharmaceutically acceptable vehicle.
- 25 The composition may be CD23-mediated side effect treatment composition. The term "CD23-mediated side effect treatment composition" can mean a pharmaceutical formulation used in the therapeutic amelioration, prevention or treatment of a CD23-mediated side effect.
- 30 The invention also provides in a thirteenth aspect, a process for making the composition according to the tenth aspect, the process comprising combining a therapeutically effective amount of a variant IgE Fc region according to the first aspect, or an antibody or a functional fragment thereof as defined in the eighth aspect, with a pharmaceutically acceptable vehicle.

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A "subject" may be a vertebrate, mammal, or domestic animal. Hence, medicaments according to the invention may be used to treat any mammal, for example livestock (e.g. a horse), pets, or may be used in other veterinary applications. Most preferably, the subject is a human being.

5

A "therapeutically effective amount" of the antibody or fragment thereof is any amount which, when administered to a subject, is the amount of agent that is needed to treat CD23-mediated side effect, or to produce the desired effect.

10

For example, the therapeutically effective amount of antibody or fragment thereof used may be from about 0.001 ng to about 1 mg, and typically from about 0.01 ng to about 100 ng. It is preferred that the amount of antibody or fragment is an amount from about 0.1 ng to about 10 ng, and most preferably from about 0.5 ng to about 5 ng.

15

A "pharmaceutically acceptable vehicle" as referred to herein, is any known compound or combination of known compounds that are known to those skilled in the art to be useful in formulating pharmaceutical compositions.

20

In one embodiment, the pharmaceutically acceptable vehicle may be a solid, and the composition may be in the form of a powder or tablet. A solid pharmaceutically acceptable vehicle may include one or more substances which may also act as flavouring agents, lubricants, solubilisers, suspending agents, dyes, fillers, glidants, compression aids, inert binders, sweeteners, preservatives, dyes, coatings, or tablet-disintegrating agents. The vehicle may also be an encapsulating material. In powders, the vehicle is a finely divided solid that is in admixture with the finely divided active agents according to the invention. In tablets, the active agent may be mixed with a vehicle having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active agents. Suitable solid vehicles include, for example calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidone, low melting waxes and ion exchange resins. In another embodiment, the pharmaceutical vehicle may be a gel and the composition may be in the form of a cream or the like.

35

However, the pharmaceutical vehicle may be a liquid, and the pharmaceutical composition is in the form of a solution. Liquid vehicles are used in preparing

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solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active agent according to the invention may be dissolved or suspended in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can
5 contain other suitable pharmaceutical additives such as solubilisers, emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium
10 carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid
15 vehicle for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions, which are sterile solutions or suspensions, can be utilized by, for example, intramuscular, intrathecal, epidural, intraperitoneal,
20 intravenous and particularly subcutaneous injection. The agent may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium.

25 The agents and compositions of the invention may be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like. The agents used
30 according to the invention can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

35

It will be appreciated that the invention extends to any nucleic acid or peptide or variant, derivative or analogue thereof, which comprises substantially the amino acid

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or nucleic acid sequences of any of the sequences referred to herein, including functional variants or functional fragments thereof. The terms “substantially the amino acid/nucleotide/peptide sequence”, “functional variant” and “functional fragment”, can be a sequence that has at least 40% sequence identity with the amino acid/nucleotide/peptide sequences of any one of the sequences referred to herein, for example 40% identity with the sequence identified as SEQ ID No:2 (i.e. wild type human IgE Fc region) or the nucleotide identified as SEQ ID No:1 (i.e. encoding wild type human IgE Fc region), or 40% identity with the polypeptides identified as SEQ ID No:3-7 (i.e. IgE Fc variant regions) or the nucleotides identified as SEQ ID No:8-12 (i.e. encoding IgE Fc variant regions), and so on.

Amino acid/polynucleotide/polypeptide sequences with a sequence identity which is greater than 50%, more preferably greater than 65%, 70%, 75%, and still more preferably greater than 80% sequence identity to any of the sequences referred to are also envisaged. Preferably, the amino acid/polynucleotide/polypeptide sequence has at least 85% identity with any of the sequences referred to, more preferably at least 90%, 92%, 95%, 97%, 98%, and most preferably at least 99% identity with any of the sequences referred to herein.

The skilled technician will appreciate how to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences. In order to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences, an alignment of the two sequences must first be prepared, followed by calculation of the sequence identity value. The percentage identity for two sequences may take different values depending on:- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that

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percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

Hence, it will be appreciated that the accurate alignment of protein or DNA sequences is a complex process. The popular multiple alignment program ClustalW (Thompson *et al.*, 1994, Nucleic Acids Research, 22, 4673-4680; Thompson *et al.*, 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW may be as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

Preferably, calculation of percentage identities between two amino acid/polynucleotide/polypeptide sequences may then be calculated from such an alignment as $(N/T) \times 100$, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating percentage identity between two sequences comprises (i) preparing a sequence alignment using the ClustalW program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:- Sequence Identity = $(N/T) \times 100$.

Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to the sequences shown in SEQ ID No's: 1, 8-12 or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x SSC/0.1% SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequences shown in SEQ ID No: 2, 3-7.

Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence described herein could be varied or changed without substantially affecting the

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sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence, which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids may be replaced with an amino acid having similar biophysical properties, and the skilled technician will know the nucleotide sequences encoding these amino acids

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying diagrammatic drawings, in which:-

Figure 1 shows NMR mapping of the CD23 and IgE interaction surfaces. (A) A small number of residues from C ϵ 3 show chemical shift perturbation upon addition of derCD23. Increasing amounts of unlabelled derCD23 were added to a 200 μ M sample of ^{15} N-labelled C ϵ 3; five spectra of the titration are overlaid (red, zero derCD23; magenta, 50 μ M; blue, 100 μ M; cyan, 150 μ M; green, 200 μ M). Insets show magnified views of the indicated regions. (B) The NMR-derived derCD23 interaction site on C ϵ 3 was mapped onto the structure of IgE-Fc (1F6A; Garman et al., 2000, *Nature* 406:259-266) and shown as surface representation. For comparison, the residues of IgE that interact with Fc ϵ RI are indicated in green. (C) The IgE interaction surface on CD23 was defined previously (Hibbert et al., 2005, *J Exp Med*

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202:751-760) and shown here as a surface representation. The interacting surfaces of IgE and CD23 are coloured according to electrostatic potential and coded such that regions with a potential $< -4 k_B T$ are red, whereas those $> 4 k_B T$ are blue (k_B , Boltzmann constant; T, absolute temperature). The IgE-CD23 interaction site is
 5 formed by complementary charged surfaces;

Figure 2 shows competition binding experiments between derCD23 and sFcεRIα for IgE-Fc. (A-D) Surface plasmon resonance and (E-F) TR-FRET-based competitive binding experiments were used to investigate the mechanisms of receptor
 10 interactions. The binding of derCD23 was tested against (A) IgE-Fc immobilized on a sensor surface and (B) IgE-Fc captured on an FcεRIα-immobilized surface; the start of the derCD23 injection is indicated with an arrow. DerCD23 binding to immobilized IgE-Fc with a K_D of $2.3 \mu M$; no measureable binding was observed for derCD23 to IgE-Fc complexed to FcεRIα. (C) The binding of IgE-Fc to immobilized
 15 derCD23 was compared with (D) the binding of a complex of IgE-Fc/sFcεRIα to the same surface; the start of the injection of the complex is indicated with an arrow. IgE-Fc binds to derCD23 with a K_D of $2.4 \mu M$, but the IgE-Fc/sFcεRIα complex does not bind to derCD23. All SPR binding experiments were performed using identical two-fold serial dilutions of ligands, from $40 \mu M$ to $78 nM$. (E) Binding between
 20 terbium-labelled derCD23 and Alexa 647-labelled IgE-Fc was measured in a solution TR-FRET assay in the presence of increasing concentrations of the αγ-fusion protein (the FcεRIα extracellular region fused to an IgG4 Fc) (Shi et al., 1997, *Biochemistry*, 36, 2112-2122); zero (black), $0.5 nM$ (red), $2.5 nM$ (blue) and $5 nM$ (green). (F) These data are also shown as a double reciprocal plot to illustrate that addition of the
 25 inhibitor results in a decrease in the apparent binding maximum (the value at $X=0$), rather than a change in the apparent binding affinity (the value at $Y=0$); this is characteristic of allosteric inhibitors;

Figure 3 shows that soluble CD23 does not cross-link IgE bound to FcεRI on mast
 30 cells. The ability of soluble CD23 to engage IgE on B cells and mast cells was tested. (A) After preincubation of IgE, the addition of anti-IgE antibody results in activation of the FcεRIα⁺ LAD-2 mast cell line, as measured by release of β-hexosaminidase. Neither monomeric derCD23 nor trimeric triCD23 is able to cross-link IgE and activate mast cells in this assay. (B) In contrast, triCD23 effectively cross-links mIgE
 35 on the surface of IgE⁺ human tonsillar B cells, resulting in activation of these cells and increased secretion of IL-4 and anti-

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CD40, and soluble CD23 was added at 1 μ M; supernatants were harvested 12 days after activation, tested for IgE levels and compared with levels for cells treated with IL-4/anti-CD40 alone (*= $p < 0.05$; **= $p < 0.01$);

5 **Figure 4** shows the temperature dependence and ionic strength dependence of the interaction between IgE-Fc and derCD23. (A) The temperature dependence of the IgE-Fc and derCD23 interaction is demonstrated by plotting the natural log of the equilibrium constant as a function of the inverse of absolute temperature (the van't Hoff plot). A linear fit of the data is shown (red line), and 95% confidence levels
10 indicated (dashed lines). The slope of this plot estimates the enthalpic changes associated with binding. At a temperature of 298K, the thermodynamic parameters of binding are as follows: $\Delta G = -32.2 (\pm 0.5) \text{ kJ mol}^{-1}$, $\Delta H = -5.0 (\pm 0.3) \text{ kJ mol}^{-1}$, $-T\Delta S = -27.2 \text{ kJ mol}^{-1}$; these values are consistent with a strong electrostatic component to binding energetics. (B) The effects of ionic strength on the binding of IgE-Fc to
15 derCD23. Binding affinities are measured over a range of ionic strengths and plotted as $\log(K_A)$ versus $\log(\text{ionic strength})$. The steep slope (-0.94) of the curve confirms a strong electrostatic contribution to binding;

Figure 5 shows the structure of the derCD23-Fc ϵ 3-4 complex. The two molecules of
20 derCD23 (light and dark blue C α traces with surfaces) bind one to each heavy chain between the C ϵ 3 (dark red and green) and C ϵ 4 domains (light red and green). The carbohydrate is shown in all-atom representation (red and yellow, without surfaces) and can be seen behind the (red) C ϵ 3 domain. The adjacent N- and C- termini of each derCD23 molecule, the former being the connection to the "stalk", the latter to
25 the "tail" region, can be seen at the extreme left and right of the figure. (The complex shown here comprises chains A (red), B (green), G (light blue) and H (dark blue));

Figure 6 shows salt bridges and hydrogen bonds at the derCD23-Fc ϵ 3-4 interface. The H-bonds associated with the four salt bridges are shown in red, additional H-
30 bonds present in all six independent interactions are shown in green, and a further H-bond present in 5/6 molecules is shown in yellow;

Figure 7 shows composite images of the derCD23 and sFc ϵ RI α complexes with Fc ϵ 3-4 to show the mutual incompatibility of their binding modes. **a)** derCD23-Fc ϵ 3-4 and sFc ϵ RI α -Fc ϵ 3-4 [PDB 1F6A] complexes superposed on their (C ϵ 4) $_2$ domain
35 pairs. The receptors are shown as surfaces (derCD23 light & dark blue; sFc ϵ RI α red)

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and the Fc ϵ 3-4 structures are shown as C α traces (in corresponding colours). The closed (derCD23-binding) and open (sFc ϵ RI α -binding) conformations of the C ϵ 3 domains may be seen. **b)** Steric clashes between the sFc ϵ RI α structure (red C α trace) and both chains of the derCD23-Fc ϵ 3-4 complex (blue) are indicated (orange surfaces). **c)** Steric clashes of both derCD23 molecules (blue C α traces) with the sFc ϵ RI α -Fc ϵ 3-4 complex (red) are indicated (green surfaces);

Figure 8 shows that sFc ϵ RI α displaces derCD23 and *vice versa* in a FRET inhibition assay. **a)** Competition of derCD23 binding to IgE-Fc (blue) and Fc ϵ 3-4 (red) by unlabeled sFc ϵ RI α . **b)** Competition of sFc ϵ RI α binding to IgE-Fc (blue) and Fc ϵ 3-4 (red) by unlabeled derCD23. (All data: n=3; error bars $\pm$ SEM);

Figure 9 shows electron density in the interface region. Electron density $2F_o - F_c$ map contoured at 1σ for key side-chains involved in salt bridges at the derCD23/Fc ϵ 3-4 interface. The side-chains shown in blue are from derCD23, those in purple from Fc ϵ 3-4;

Figure 10a) The six independent Fc ϵ 3-4 heavy chains superposed on their C ϵ 4 domains, showing the variation in the relative positions of the C ϵ 3 domains. **b)** The six independent derCD23 molecules superposed, showing virtually no overall structural variation;

Figure 11 shows a comparison of Fc ϵ 3-4 heavy chains taken from various Fc ϵ 3-4 and IgE-Fc structures superposed by their C ϵ 4 domains upon the set of six independent heavy chains of the derCD23-Fc ϵ 3-4 complex (shown lightly coloured). **a)** Free IgE-Fc (light and dark red) [PDB 2WQR; 28], **b)** sFc ϵ RI α -IgE-Fc complex (light and dark cyan) [PDB 2Y7Q; 21], **c)** sFc ϵ RI α -Fc ϵ 3-4 complex (light and dark purple) [PDB 1F6A; 20], **d)** Single chain of free Fc ϵ 3-4 (green), the most closed structure observed to date [PDB 3HA0; 27]; and

Figure 12 shows the superposition of apo- and calcium-bound derCD23 structures. The apo-CD23 (dark blue) and calcium-bound CD23 (dark red) structures [PDB 2H2R, 2H2T; 40] superposed upon the six derCD23 molecules from the Fc ϵ 3-4 complex (lightly coloured), showing differences in loops 1 and 4. The Ca²⁺ ion is

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shown (red sphere); note the disordered loop 4 between residues Ser252 and Asp258 in the calcium-bound structure.

Examples

5 Example 1

Materials and Methods

Protein expression and purification: Recombinant human IgE-Fc (comprising domains C ϵ 2-C ϵ 4)(Wan et al., 2002, *Nat Immunol* 3:681-686), the $\alpha\gamma$ -fusion protein
10 (the Fc ϵ RI α extracellular region fused to an IgG4 Fc) (Shi et al., 1997, *Biochemistry* 36, 2112-2122), soluble Fc ϵ RI α (Price et al., 2005, *J Biol Chem* 280, 2324-2330), derCD23 (Hibbert et al., 2005, *J Exp Med* 202, 751-760) and the C ϵ 3 domain (Price et al., 2005, *J Biol Chem* 280, 2324-2330) were each produced and purified as described previously. Primers for site-directed mutagenesis were obtained from
15 Sigma-Genosys (Sigma Lifescience), and mutagenesis was performed using the QuikChange II Kit (Stratagene) according to the manufacturer's instructions. Mutant constructs were expressed and purified using the same methods as the wildtype proteins. MAb 7.12 was produced from a B cell hybridoma (Kanowith-Klein et al., 1988, *Clin Immunol Immunopathol* 48, 214-224), and was a kind gift of Dr. Rebecca
20 Beavil (King's College London, UK).

NMR spectroscopy: NMR spectroscopy was performed on protein samples in a buffer containing 25mM Tris, 125 mM NaCl, 4mM CaCl₂, pH 6.8, at protein concentrations between 120 and 900 μ M. Data were collected at 25°C on Bruker spectrometers
25 equipped with CryoProbes operating at 500 and 700 MHz. For chemical shift perturbation experiments, unlabelled derCD23 ligands were concentrated to 2mM and then added in small aliquots to samples of 200 μ M ¹⁵N-labelled C ϵ 3 until saturation was seen. The NMR chemical shifts of the urea denatured and native state C ϵ 3 domain are available from the BioMagResBank database under accession
30 numbers 18482 and 18483.

Surface plasmon resonance: All experiments were performed on a Biacore T100 instrument (GE Healthcare), essentially as described previously (Hibbert et al., 2005, *J Exp Med* 202, 751-760; Holdom et al., 2011, *Nat Struct Mol Biol* 18, 571-576).
35 Specific binding surfaces were prepared using standard amine coupling methods for derCD23 and the $\alpha\gamma$ -fusion protein, whereas IgE-Fc was biotinylated (one biotin per

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molecule) and captured on a streptavidin surface. Coupling densities were kept low (<100 RU) to minimize potential avidity effects. Ligands in HBS (10mM HEPES, pH 7.4, 150mM NaCl, 4mM CaCl₂, 0.005% surfactant p20) were injected at 25 µl/min with a 1-min association phase followed by a 15-min dissociation phase.

5 Thermodynamic measurements were performed at 288K, 293K, 298K and 303K. For the sandwich binding experiments, approximately 90RU of IgE-Fc was captured on an $\alpha\gamma$ -fusion protein surface during a 1-min injection of a 10nM IgE-Fc sample; after a 3-min stabilization period, 0-100µM derCD23 was injected for 1-min followed by a 15-min dissociation phase. All measurements were done independently at least
10 twice, using standard double reference subtraction methods for data analysis (Myszka, 1999, *J Mol Recognit* 12, 279-284). Curve fitting and other analyses were performed using MicroCal Origin 8.0 software (OriginLab Corporation).

FRET assay: Inhibition assays were performed by competing 1µM terbium-chelate
15 labelled derCD23 and 0-20µM Alexa 647-labelled IgE-Fc with a range of concentrations of unlabelled $\alpha\gamma$ -fusion protein. Protein mixtures were prepared in Lanthascreen buffer (Invitrogen) in triplicate, in 384-well plates (Greiner Bio-One), and equilibrated overnight at room temperature. FRET measurements were made on an Artemis plate reader (Berthold Technologies). TR-FRET ratios were calculated for
20 each well as the emission of acceptor at 665nm divided by the emission of donor at 620nm and then multiplied by 10,000. Apparent K_D and B_{max} values were derived from non-linear curve fitting of inhibition titrations, and data are presented as double reciprocal plots to highlight the differences in apparent B_{max} values upon addition of the inhibitor.

25 Mast cell degranulation assay: The human mast cell line LAD-2 (NIH) was cultured in Stem Pro-34 SFM medium (Gibco) supplemented with L-glutamine (2 mM), penicillin (10 U/ml), and streptomycin (1 µg/ml; all from Life Technologies). LAD-2 cells were suspended in culture medium at 1.6x10⁶ cells/ml and 50µl aliquots were
30 placed in v-bottomed 96-well plates (Greiner Bio-One). Cells were primed by addition of 2.5nM IgE (NIBSC) or a buffer-only control for one hour, before addition of cross-linking reagents. Polyclonal rabbit anti-human IgE (Dako) was added at 20nM and soluble CD23 constructs at 0.1, 1 and 10µM, and incubated for 1 hour at 37°C. Supernatants were harvested and tested for β -hexosaminidase release,
35 measured fluorometrically as described previously (Hammond, 2006, *In Cell Biology*, J.E. Celis, editor. Elsevier. Amsterdam). Controls included cells treated with wash

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buffer plus 1% Triton-X for total release, with buffer-only to measure background release, typically about 10% of total release, with 2.5nM IgE-only, and with 10 μ M CD23-only. The level of degranulation measured for Triton-X treated cells was defined as 100% release and all samples were defined relative to that.

5

B cell activation assays: Human tonsillar B cells were cultured in 24-well plates (Nunc) at 5 x 10⁵ cells/ml in RPMI with penicillin (100IU/ml), streptomycin (100 μ g/ml), glutamine (2mM) (Invitrogen), 10% FCS (Hyclone, Perbio Biosciences Ltd), insulin (5 μ g/ml) and transferrin (35 μ g/ml) (Sigma-Aldrich). Cells were
10 activated with IL-4 (200IU/ml) (R&D Systems), anti-CD40 antibody (1 μ g/ml) (G28.5; ATCC), and either 1 μ M derCD23 or 1 μ M triCD23. Supernatants were harvested on day 12 for IgE measurements. IgE ELISA assays were performed as described previously (Cooper et al., 2012, *J Immunol* 188, 3199-3207), using
15 Maxisorp plates (Nunc) coated with polyclonal mouse anti-human IgE (1:7000) (Dako), in pH 9.8 carbonate buffer (0.2M Na₂CO₃, 0.2M NaHCO₃). Unbound sites were blocked with 2% milk powder in PBS + 0.05% Tween20 (Sigma-Aldrich). Binding was detected by mouse anti-human IgE-HRP (1:1000) (Dako) in 1% milk powder in PBS-T for 2 hours at 37°C. Standard curves were derived using human serum IgE (NIBSC), with a minimal detectable concentration of about 2ng/ml.

20

Results and discussion

In an earlier study, the inventors identified the IgE binding site on CD23 using NMR chemical shift perturbation studies (Hibbert et al., 2005, *J Exp Med* 202, 751-760). In this work, the inventors have now performed the reciprocal NMR binding
25 experiment, mapping the interaction site of CD23 onto the C ϵ 3 domain from IgE. Using an approach described by Schulman *et al.* (Schulman et al., 1997, *Nat Struct Biol* 4, 630-634), they then assigned the backbone resonances of the molten globule C ϵ 3 domain by first performing resonance assignments of C ϵ 3 denatured in 6M urea and then, through gradual titration of buffer conditions, tracking those resonances to
30 the native state C ϵ 3 domain. Next, the inventors titrated unlabelled monomeric CD23 protein (derCD23) against an ¹⁵N-labelled C ϵ 3 sample and used the assigned NMR spectra to identify residues that were affected by addition of ligand. A small number of C ϵ 3 residues showed peak shifting and line broadening during the derCD23 titration, consistent with an interaction characterized by fast/intermediate
35 exchange kinetics (see Fig. 1A).

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When mapped onto the surface of the protein, the identified residues from three discontinuous sequences (amino acids 405-407, 409-411 and 413 from the E-F helix of the C ϵ 3 domain of IgE, amino acids 377-380 from the C-D loop, and residue 436 from the C-terminal region) form a contiguous surface representing the binding site on C ϵ 3 for CD23 (Figure 1B). This region is at the end of the C ϵ 3 domain, near to the interface with C ϵ 4, in contrast to the interaction site for Fc ϵ RI, which is at the other end of C ϵ 3 near the interface with C ϵ 2 (Garman et al., 2000, *Nature* 406, 259-266; Holdom et al., 2011, *Nat Struct Mol Biol* 18, 571-576) (Fig. 1B).

The identification of this CD23 interaction site on IgE provides a structural explanation for the experimentally observed 2:1 (CD23:IgE) stoichiometry, as the dimeric IgE-Fc can bind to two separate CD23 lectin head domains. The two CD23 interactions were shown to have slightly different binding affinities and thermodynamic characteristics, as was also observed for the Fc α RI-IgA interaction. The two binding affinities imply an asymmetry of the two CD23 binding sites, which may possibly be allosterically induced. CD23's capacity for inducing a conformational change in IgE is discussed further below.

In support of the NMR-based interaction site mapping, the inventors then analysed the binding characteristics of the IgE-CD23 interaction and performed extensive site-directed mutagenesis of residues at the binding interface. Surface plasmon resonance (SPR) based measurements of binding thermodynamics indicate an interaction with a strongly favorable entropic contribution (at 298K, $\Delta G = -32.2 (\pm 0.5) \text{ kJ mol}^{-1}$, $\Delta H = -5.0 (\pm 0.3) \text{ kJ mol}^{-1}$, $-T\Delta S = -27.2 \text{ kJ mol}^{-1}$) (Figure 4A). Binding experiments performed over a range of different ionic strengths gave a measure of the contributions of electrostatic forces to the overall binding energies; a plot of $\log(K_A)$ versus $\log(\text{ionic strength})$ shows an unusually steep slope of -0.94 (Fig. 4B) (in comparison, the same analysis of the IgE-Fc-sFc ϵ RI α interaction gives a slope of -0.13). Both of these measurements are consistent with the IgE-CD23 binding event being strongly electrostatic in character.

Following the NMR mapping of interaction epitopes for both proteins, the inventors used site-directed mutagenesis to validate the interaction site in the context of the full IgE-Fc construct and to define the energetic contributions of individual residues. Ten mutants from derCD23 and eleven mutants from IgE-Fc (domains C ϵ 2-4) were produced, purified and characterized; their binding affinities were measured using an

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SPR assay (Hibbert et al., 2005). Table 1 summarizes the results of the site-directed mutagenesis studies.

Table 1 - Effects of mutations on the IgE-CD23 interaction

IgE-Fc mutation	K _D (μM)	ΔΔG (kJ mol ⁻¹)	derCD23 mutation	K _D (μM)	ΔG (kJ mol ⁻¹)
wildtype	2.3	-	wildtype	2.3	-
D409A	26.3	+6.0	D227A	30.9	+6.4
E412A	24.2	+5.8	E257A	26.7	+6.1
R376A	19.7	+5.3	R224A	26.2	+6.0
K380A	13.3	+4.3	R188A	25.0	+5.9
K435A	5.0	+1.9	Y189A	15.6	+4.7
K352A	3.8	+1.2	K276A	10.9	+3.9
R351A	2.6	+0.3	L226A	6.6	+2.6
D347A	2.5	+0.2	D236A	5.8	+2.3
P439A	2.5	+0.2	D192A	4.3	+1.6
Q535A	2.5	+0.2	E265A	2.5	+0.2
Q538A	2.4	+0.1			

5

Mutations on both proteins that affect binding are entirely consistent with the NMR-defined interaction sites. Charged residues have the largest energetic effect on binding. CD23 mutations D227A, E257A, R224A and R188A all show a change in binding free energy (ΔΔG) of about +6 kJ mol⁻¹ (Table 1). Uncharged residues also contribute to the binding energy; a prominently exposed tyrosine residue (Y189) in the center of CD23's IgE binding site makes a substantial contribution to binding energy. The CD23 binding surface on IgE is also predominantly electrostatic, with residues D409, E412, R376 and K380 showing the largest effects on CD23 binding energetics.

15

An earlier study had implicated IgE residue K352 from the A-B helix as part of the binding interface. However, the inventors' NMR perturbation experiments indicate that the A-B helix is near the site of interaction, but in the assay reported herein that directly measures binding affinity, a K352A mutation had only a very small effect on CD23 binding (see Table 1). The high-resolution crystal structure of the IgE-CD23 complex indicates that K352 is not part of the binding site. The inventors postulate that it is likely that the effects of the non-conservative K352E mutation previously

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observed resulted from local conformational changes rather than a direct effect from K352 on CD23 binding. Because the NMR data indicated a site on C ϵ 3 very near to the C ϵ 4 interface (Fig. 1B) and because binding sites from several other immunoglobulin-receptor interactions involve sites analogous to the C ϵ 3-C ϵ 4 interface, the inventors also made a pair of mutations in the F-G loop of the C ϵ 4 domain, close to the CD23 binding site in C ϵ 3. However, neither Q535A nor Q538A appear to affect CD23 binding, leading the inventors to believe that CD23's binding surface on IgE is largely restricted to residues from C ϵ 3.

Earlier studies indicated that soluble CD23 can compete with Fc ϵ RI binding, and this was attributed to steric competition for an overlapping binding site within the C ϵ 3 domain. However, the data described herein show that the CD23 and Fc ϵ RI binding sites are spatially distinct and suggest that the mechanism of mutual inhibition must be allosteric in nature. The inventors performed a set of competitive binding assays to confirm this experimentally. Firstly, using an SPR assay, they showed that derCD23 can bind to IgE-Fc immobilized to an SPR chip but cannot bind to IgE-Fc captured by immobilized Fc ϵ RI α (Fig. 2A,B); a positive control, a Fab fragment of the anti-IgE antibody 7.12, directed against the C ϵ 2 domain, did bind to Fc ϵ RI α -captured IgE-Fc (data not shown). Secondly, they showed that IgE-Fc can bind to immobilized derCD23, but an IgE-Fc-sFc ϵ RI α complex cannot bind to derCD23 (Fig. 2C,D). These data indicate that CD23 and Fc ϵ RI interactions with IgE are mutually inhibitory. Finally, they also tested the ability of the receptors to compete for binding to IgE in a solution TR-FRET experiment (Selvin, 2002, *Annu Rev Biophys Biomol Struct* 31, 275-302). This assay can be performed under equilibrium binding conditions, allowing a different set of mechanistic properties to be tested than in the kinetic SPR experiments. Under equilibrium conditions, different inhibition patterns are observed for competitive and allosteric inhibitors. A competitive inhibitor affects the apparent binding affinity, with inhibitor I reducing the apparent affinity by a ratio of $(1+[I]/K_I)$; whereas an allosteric inhibitor affects the apparent $B_{\max}$ of the interaction without changing the apparent K_D (Fersht, 1999, *Structure and Mechanism in Protein Science*. New York, W.H. Freeman.). Using Fc ϵ RI α as the inhibitor of the IgE-Fc-derCD23 interaction results in a decrease of apparent $B_{\max}$ without affecting the apparent K_D of the interaction (Fig. 2E,F). These experiments confirm mutual inhibition by the two IgE receptors and offer experimental evidence that an allosteric mechanism is involved.

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Given the location of the CD23 binding site, the most obvious mechanism for allostery is a conformational change around the interface between the C ϵ 3 and C ϵ 4 domains. Crystal structures of IgE-Fc and IgE-Fc-Fc ϵ RI α complexes indicate that the C ϵ 3 domains can exist in "open" and "closed" states, with only an open state being
5 capable of binding Fc ϵ RI. A detailed study of the open and closed states concluded that it is the motions around the C ϵ 3 A-B helix, sitting at the C ϵ 3-C ϵ 4 interface, that control the orientation of the two C ϵ 3 domains.

Soluble trimeric CD23 has been shown to bind to and cross-link membrane IgE on B
10 cells, resulting in B cell activation. However, it is essential that trimeric CD23 not cross-link IgE bound to Fc ϵ RI on the surface of mast cells. If this were to occur then high levels of CD23 would result in mast cell activation in the absence of allergens. The data from binding experiments (Fig. 2B) predict that soluble CD23 cannot directly cross-link IgE bound to Fc ϵ RI on mast cells. The inventors tested this
15 prediction in a mast cell degranulation assay using the Fc ϵ RI⁺ LAD-2 human mast cell line. In this assay, cells are first primed by adding IgE, followed by addition of potential cross-linking reagents and measurement of release of the mast cell granule-associated enzyme β -hexosaminidase. An anti-IgE antibody results in Fc ϵ RI-mediated activation of the mast cell and robust β -hexosaminidase release, but the
20 addition of either the monomeric derCD23 or a trimeric CD23 construct (triCD23) fails to induce mast cell degranulation (Fig. 3A). In contrast, trimeric CD23 effectively cross-links IgE on B cells, resulting in activation of these cells and increased production of soluble IgE (Fig. 3B).

25 Immunoglobulins have evolved two separate sites for binding to receptors. One site is near the hinge region in IgG and at the C ϵ 2-C ϵ 3 interface in IgE, while the other is at the interface of the C-terminal domain and the penultimate domain: the C ϵ 3-C ϵ 4 interface in IgE. A mechanism of communication has evolved within the IgE molecule between these two distant sites to prevent simultaneous engagement of
30 CD23 and Fc ϵ RI. This may be a unique property of IgE. Since IgE and CD23 both exist in membrane bound and soluble forms, and soluble Fc ϵ RI α has also recently been shown to exist at functionally relevant concentrations, there is considerable potential for receptor cross-regulation. Mutually exclusive receptor binding assures independent functions for IgE-Fc ϵ RI and IgE-CD23 interactions.

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IgE is a clinically important drug target. An anti-IgE antibody (omalizumab) is an effective therapy, currently used in the treatment of moderate to severe asthma that is not controlled by corticosteroids. Omalizumab binds to the C ϵ 3 domain of IgE and competitively inhibits Fc ϵ RI binding, although its *in vivo* activity relies on more than
5 just inhibition of this interaction. Results presented here demonstrate that IgE is amenable to allosteric inhibition, an approach that may have significant advantages over competitive inhibition, and lay the foundation for the development of allosteric modulators of IgE-receptor interactions.

10 Summary

IgE, the antibody that mediates allergic responses, acts as part of a self-regulating protein network. Its unique effector functions are controlled through interactions of its Fc region with two cellular receptors, Fc ϵ RI on mast cells and basophils and CD23 on B cells. IgE cross-linked by allergen triggers mast cell activation via Fc ϵ RI, while
15 IgE-CD23 interactions control IgE expression levels. The inventors have determined the CD23 binding site on IgE, using a combination of NMR chemical shift mapping and site-directed mutagenesis. They have shown that the CD23 and Fc ϵ RI interaction sites are at opposite ends of the C ϵ 3 domain of IgE, but that receptor binding is mutually inhibitory, mediated by an allosteric mechanism. This prevents
20 CD23-mediated cross-linking of IgE bound to Fc ϵ RI on mast cells and resulting antigen-independent anaphylaxis. The mutually inhibitory nature of receptor binding provides a degree of autonomy for the individual activities mediated by IgE-Fc ϵ RI and IgE-CD23 interactions.

25 It will be appreciated that by inhibiting binding between the Fc region on IgE and CD23 (which has been clearly demonstrated herein), one can be confident about inhibiting the downstream functional activity and avoiding CD23-mediated side effects, which are often experienced when using therapeutic IgE antibodies.

30 Example 2

Materials and Methods

Protein expression and purification: Recombinant human derCD23 (Ser156 – Glu298) was expressed, refolded and dialyzed into 25 mM Tris-HCl pH 7.5
35 ('purification buffer') as previously described (Hibbert, 2005, *J. Exp. Med.*, 202, 751-760). It was purified on a heparin-sepharose column (GE Healthcare) pre-

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equilibrated with purification buffer, eluting with 25 mM Tris-HCl pH 7.5, 200 mM NaCl. Fractions were pooled, concentrated to 1 ml and loaded onto a HiLoad 16/60 Superdex G75 column (GE Healthcare), pre-equilibrated and subsequently washed with purification buffer. Folding was assessed by 1D-¹H NMR at 500 MHz (large
5 dispersion and strong signals of methyl groups between 1.0 and -1.0 ppm). Human IgE-Fc (N265Q, N371Q) was expressed in NSO cells and purified by affinity chromatography with sFcεRIα-IgG₄-Fc fusion protein as previously described (Shi, 1997, *Biochemistry*, 36, 2112-2122). The genes for recombinant human Fcε3-4 (Cys328-Lys547, with N-terminal ADP) and sFcεRIα-Cys-His (Val1-Lys176, with C-
10 terminal cysteine and His₆ tag) were synthesized by DNA2.0 and cloned as HindIII/EcoRI fragments into proprietary mammalian expression vectors. The DNAs were transiently transfected into HEK293 cells using 293-fectin (Life Technologies) according to the manufacturers' instructions and the supernatants were harvested 6 days post transfection. Human Fcε3-4 was purified by cation
15 exchange chromatography on a SPHP matrix (GE Healthcare) in 50 mM NaOAc buffer pH 6.0, followed by gel filtration on a Superdex S200 matrix (GE Healthcare) in PBS pH 7.4. Human sFcεRIα-Cys-His was purified on a Ni-NTA column (Qiagen) followed by gel filtration on a Superdex S200 matrix (GE Healthcare) in PBS pH 7.4 and stored under nitrogen to prevent reactivity of the free cysteine residue.

20

Crystallization and data collection: Fcε3-4 was concentrated to 20 mg/ml, and derCD23 to 18 mg/ml, in 25 mM Tris-HCl pH 7.5, 20mM NaCl, 0.05% sodium azide ('crystallization buffer'). The complex was formed with 0.4 mM derCD23 (6.2 mg/ml), 0.2 mM Fcε3-4 (10 mg/ml) and 4 mM CaCl₂, diluted with equal volume of
25 3% PEG 8,000, 0.1 M Tris-HCl pH 7.5 as the precipitant. Crystals grew to ~400 μm within 8 days at 295 K, were flash-cooled to 100K (using 14% PEG 8,000, 0.1 M Tris-HCl pH 7.5, 30% PEG 200) and data collected at beamlines I02 and I04, Diamond Light Source (Harwell, U.K.).

30 FRET assay: Labeling of the proteins is described in *SI Materials and Methods*. Inhibition assays were performed by competing 5 % of 1 μM terbium labeled derCD23 and 5 % of 5 μM Alexa Fluor 647 labeled IgE-Fc or Fcε3-4 with a dilution series of unlabeled sFcεRIα-Cys-His. Assays were conducted in 384 well hi-base, white plates (Greiner BioOne) using Lanthascreen buffer (Invitrogen) as a diluent. The plate was
35 left to incubate for 1 hr at room temperature with shaking and read by the Artemis plate reader (Berthold Technologies). TR-FRET ratios were then calculated for each

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well as the emission of acceptor at 665 nm divided by the emission of donor at 620 nm multiplied by 10^4 . Data were analyzed using *GraphPad Prism 5*. Similarly, 2 nM terbium labeled sFc ϵ RI α and 10 nM Alexa Fluor 647 labeled IgE-Fc or Fc ϵ 3-4 were competed with a dilution series of unlabeled derCD23. Assays were conducted as described above with the exception of an overnight incubation. Positive controls for the two experiments were provided by displacement of labeled sFc ϵ RI α by unlabeled sFc ϵ RI α , and labeled derCD23 by unlabeled derCD23; these defined the start- and end-point values for each titration, which were treated as zero and 100% inhibition for calculation of the “% inhibition” values for displacement of one receptor by the other (Figs. 8a & b).

Crystallization: Crystals with a different morphology were also found after 6 days with 12% PEG 4,000, 0.1 M Tris-HCl pH 8.75, 0.2 M sodium acetate trihydrate as precipitant after streak seeding. Diffraction data were collected to 3.6Å and the crystal form was found to be the same as that reported in Table 2.

Crystallographic data collection and structure determination: Indexing, integration and merging of data to 3.1Å resolution was carried out with the *HKL2000* suite of programs (Otwinowski, 1997, *Methods Enzymol.*, 276, 307-326). Nine previously determined Fc ϵ 3-4 dimers (Wurzburg & Jardetzky, 2009, *J. Mol. Biol.*, 393, 176-190) were used as search models in molecular replacement. However, due to the high degree of conformational flexibility of the two Fc ϵ 3-4 chains (Wurzburg & Jardetzky, 2009, *J. Mol. Biol.*, 393, 176-190), fourteen additional dimers were manually generated from crystallographically independent chains and were also used as search models. Solutions were only found using the “closed” conformations of the Fc ϵ 3-4 dimer. For example, using a dimer generated from chain A of PDB 3H9Z, two copies of the Fc ϵ 3-4 dimer were identified in the asymmetric unit using *PHASER* (MacCoy, 2007, *J. Appl. Crystallogr.*, 40, 658-674). Subsequently, four derCD23 (PDB 2H2R) molecules were located. A third derCD23–Fc ϵ 3-4 complex could only be found by searching with one of the previously located complexes.

Due to the high degree of NCS, reflections were selected for the R_{free} set in thin resolution shells (Fabiola, 2006, *Acta Crystallogr D*, 62, 227-238) using *SFTOOLS* (Winn, 2011, *Acta Crystallogr D*, 67, 235-242). Iterative cycles of refinement using *PHENIX* (Adams, 2011, *Methods*, 55, 94-106), *REFMAC5* (Murshudov, 2011, *Acta Crystallogr D*, 67, 355-367) and *BUSTER-TNT* (Bricogne, BUSTER-TNT, Global

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Phasing Ltd.) alternated with manual model building with *COOT* (Emsley, 2010, *Acta Crystallogr D*, 66, 486-501). To minimize bias, the model was built into $2F_o - F_c$ composite omit, $2F_o - F_c$ and $F_o - F_c$ electron density maps. Initially during refinement, tight NCS restraints were used. These were gradually relaxed, and finally

5 Local Structure Similarity Restraints (LSSR or 'local NCS') (Murshudov, 2011, *Acta Crystallogr D*, 67, 355-367) were applied. TLS groups (Painter, 2008, *Acta Crystallogr D*, 62, 439-450) were generated using the *TLSMD* web server (Painter, 2006, *J Appl Crystallogr*, 39, 109-111). Carbohydrate atoms and a single water molecule were subsequently incorporated into the structure. No electron density was

10 observed for Fcε3-4 residues 363 – 364 of chain B; 367 – 371 and 421 -428 of chain C; 367 – 370, 419 – 422, 429 – 430, 479 – 480 and 517 - 519 of chain E; and derCD23 residues 256 – 257 of chains G, H, I, J and K. Therefore, these residues were not built into the model. Refinement statistics are shown in Table 2.

15 Labeling of proteins for FRET assay: IgE-Fc and Fcε3-4 were labeled with acceptor fluorophore by reacting 4 mg/ml protein in 100 mM sodium bicarbonate, 50 mM NaCl, pH 9.3, with a 5-fold molar excess of Alexa Fluor 647 succinimidyl ester (Invitrogen). After 1 hr incubation at room temperature with agitation, excess unreacted fluorophore was removed by dialyzing into HBS (10 mM Hepes, 150 mM

20 NaCl, 4mM CaCl₂, pH 7.4). derCD23 and the sFcεRIα-Cys-His mutant were labeled with donor fluorophore. derCD23 at 3.5 mg/ml in 100 mM Hepes, 125 mM NaCl, pH 8.3 was reacted with a 5-fold molar excess of terbium chelate isothiocyanate (Invitrogen) for a 3 hr incubation at room temperature with agitation. Excess fluorophore was removed by dialyzing into HBS. sFcεRIα-Cys-His at 5 mg/ml in PBS

25 (20 mM phosphate buffer saline, pH 7.4) was reacted with a 10-fold molar excess of terbium chelate maleimide (Invitrogen) overnight at 4°C *via* thiol coupling.

Results

Overall topology of the complex

30 The structure of the complex was determined at 3.1Å resolution and reveals one derCD23 molecule bound to each heavy chain at the interface between Cε3 and Cε4, making contact with residues from both domains (Fig. 5 and Table 2; Fig. 9 shows the electron density map at the interface).

Table 2 - Crystallographic data collection and refinement statistics

Data processing statistics

Beamline	Io4, Diamond Light Source
Wavelength (Å)	0.9763
Space group	$P2_12_12_1$
Unit cell parameters (Å)	$a = 62.89, b = 110.75, c = 376.56$
Resolution range (Å)*	50.0 – 3.10 (3.21 – 3.10)
Observations	162,066
Unique reflections	48,290
Average redundancy	3.6 (4.0)
Completeness (%)	92.8 (94.7)
Wilson B factor (Å ²)	117
$I/\sigma(I)$	14.5 (1.68)
$R_{\text{merge}}^{\dagger}$	0.086 (0.588)

Refinement statistics

Resolution range (Å)	29.9 – 3.1
Total No. of reflections	48,257
No. of working reflections	45,820
No. of test reflections	2,437
R factor [‡]	0.2649
R_{working}	0.2640
R_{free}	0.2831
No. of atoms	
Protein	16,107
Others [§]	389
R.m.s. bond-length deviation (Å)	0.041
R.m.s. bond-angle deviation (°)	1.29
Mean B factor (Å ²)	111
Main chain	107
Side Chain	115
Others [§]	131
R.m.s. backbone B factor deviation [¶]	2.6
Ramachandran statistics (%) ^e	
Favored	92.9
Allowed	100
Outliers	0

* Values in parentheses are for the outer resolution shell.

$$^{\dagger} R_{\text{merge}} = \sum |I_{\text{obs}} - \langle I \rangle| / \sum \langle I \rangle$$

$$^{\ddagger} R \text{ factor} = \sum_{hkl} |F_o(hkl)| - |F_c(hkl)| / \sum_{hkl} |F_o(hkl)|$$

5 § Carbohydrate and one water molecule.

¶ R.m.s. deviation between *B* factors for bonded main-chain atoms.

^e As defined by MolProbity (Chen, 2010, *Acta Crystallogr D* 66:12-21). .

The crystal form contained three independent copies of the complex in the
 10 asymmetric unit and the six heavy chains (labeled A to F) bound to six derCD23
 heads (labeled G to L) showed essentially identical modes of interaction, exemplified
 by the structure depicted (Fig. 5; chains A, B, G & H). The angle between the Cε3 and
 Cε4 domains varies only slightly between the six heavy chains (Fig. 10a) and there are
 virtually no differences between the six derCD23 head domains (Fig. 10b). All three
 15 complexes thus display approximate two-fold symmetry, although one (consisting of
 chains C, D, I & J) includes the two “extremes” in terms of the angle between the Cε3
 and Cε4 domains, but these differ by only 7 degrees. On all six heavy chains, electron
 density was present for five N-linked sugar units at Asn394 ([N-
 acetylglucosamine]₂[Mannose]₃; Fig. 5), and an additional two mannose residues
 20 were visible on chain C.

Flexibility in the Cε3-Cε4 inter-domain angle has been well-documented through
 studies of unliganded Fcε3-4 structures in different crystal forms, as well as the
 unliganded IgE-Fc structure and the sFcεRIα complexes with both Fcε3-4 and IgE-
 25 Fc. Amongst all of these structures the inter-domain angle varies over a range of
 approximately 25 degrees between the most “open” (in the sFcεRIα complexes) and
 the most “closed” (chain D in the derCD23 complex reported here) (Fig. 11a-d).
 Previously, the most closed conformation had been seen in one of the unliganded
 Fcε3-4 structures (chain D of PDB 3HAO), and the conformations seen in the
 30 derCD23 complex all range within 3 or 4 degrees of this closed conformation (Fig.
 11d).

Nature of the IgE/CD23 interface

The 2:1 stoichiometry agrees with earlier measurements in solution by analytical
 35 ultracentrifugation, recorded for both Fcε3-4 and IgE-Fc, but direct contact with Cε4
 was not anticipated by previous mutagenesis or peptide inhibition studies. Three salt

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bridges between Fc ϵ 3-4 and derCD23 (Asp409-Arg188; Glu412-Arg188; Glu412-Arg224) and a potential fourth (Glu414-His186 if protonated), together with four additional hydrogen bonds (Arg376-Tyr189; Asp409-Tyr189; Arg440-Ser254, both side-chain and main-chain) dominate the interaction (Fig. 6 and Fig. 9). In the four salt bridges, all negative charges reside on IgE, all positive charges on CD23. Arg440, while H-bonding to Ser254 of derCD23, retains its intra-chain salt bridge interaction with Glu529 of C ϵ 4 that is seen in other, unliganded Fc ϵ 3-4 structures. These salt bridging and hydrogen bonding residues of IgE are not conserved in the other human antibody classes (Fig. 12), consistent with the specificity of CD23 for IgE.

Remarkably few additional residues make well-defined van der Waals contacts in all six interfaces (Ile411, Gly413, Pro439, Glu529, Gln535 in Fc ϵ 3-4; Trp184, Val185, Asp227 in derCD23) and the surface complementarity is poor (calculated Sc values for the six interfaces range from 0.63 to 0.71); indeed, there is a substantial cavity at the interface between Glu412 (in Fc ϵ 3-4), Arg224 and the Cys259-Cys273 disulphide bridge (in derCD23). The buried surface area for each interaction ranges from 860 to 890Å² for all except one (chains F & L) at 920Å²; in this latter case the derCD23 loop at residues Ser256 and Glu257 is not as disordered as it is in the other five independent views of the interaction. The C ϵ 3 domain dominates the interface with 63% of the contact area, a further 25% involves the linker region (residues 437 to 440) and C ϵ 4 contributes 12%. All of the salt-bridge and hydrogen bond interactions involve the C ϵ 3 domain with the exception of Arg440 in the linker region, while C ϵ 4 contributes only van der Waals interactions. Earlier studies had implicated the AB loop of C ϵ 3, and residue Lys352 in particular, in CD23 binding, but this loop is not directly involved at all and Lys352 makes no contact with CD23; any effects of mutagenesis in this loop must therefore be indirect. It is the EF loop/helix of the C ϵ 3 domain that is centrally placed at the interface (Fig. 6). Surprisingly, no Ca²⁺ ions were found bound to derCD23 in the complex; the role of Ca²⁺ in the CD23-IgE interaction is discussed below.

Conformational change upon CD23 binding to IgE-Fc

In the free state, IgE-Fc adopts an asymmetrically bent structure with the (C ϵ 2)₂ domain pair folded back against one of the C ϵ 3 domains, but although the two C ϵ 3-C ϵ 4 pairs have different inter-domain angles, both are more open than that seen in the derCD23-Fc ϵ 3-4 complex (Fig. 11a). To assess whether the presence of the C ϵ 2

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domains might be expected to affect derCD23 binding, the (Cε2)₂ domain pair was modeled onto the derCD23-Fcε3-4 complex. This was achieved by superimposing the Cε3 domain of free IgE-Fc that contacts the (Cε2)₂ domain pair, onto the Cε3 domain (chain A) of the derCD23-Fcε3-4 complex. One of the derCD23 molecules (chain G) lay very close to the Cε2 domains, with a minor rearrangement of the N-terminal residues of Cε2 required to prevent a steric clash. Contact between side chains of this derCD23 molecule and Cε2 in a complex with IgE-Fc (or indeed IgE) certainly cannot be ruled out; the other bound derCD23 molecule (chain H) however, lay far from the Cε2 domains. Modeling of the Cε2 domains onto the Fcε3-4 complex also revealed that the AB loop of Cε2 (chain A) would have to adopt a slightly different conformation to avoid clashing with Cε3 (chain B) in the very closed conformation that this Cε3 domain adopts in the complex.

Mutual exclusion of FcεRI and CD23 binding to IgE-Fc

The closed conformation for the Cε3 domains in the derCD23 complex is clearly incompatible with FcεRI binding. This may be seen by superimposing the derCD23-Fcε3-4 and the sFcεRIα-Fcε3-4 (Garman, 2000, *Nature*, 406:259-266) complexes on their (Cε4)₂ domain pair, and noting the very different orientations of the Cε3 domains (Fig. 7a). The steric clashing of sFcεRIα with the Cε3 domains in their CD23-bound conformation, and of both derCD23 molecules with the Cε3 and Cε4 domains in their sFcεRIα-bound conformations, is clear from the superposition of the complexes upon one or other of the Cε3 domains (Figs. 7b & c). Thus IgE cannot bind both FcεRI and CD23 simultaneously: binding of sFcεRIα causes conformational changes that are incompatible with binding of *either* derCD23 molecule, and the binding of *either* derCD23 molecule ensures that the two sub-sites required for high-affinity binding of FcεRI are not both accessible.

This surprising conclusion was verified experimentally in solution using acceptor fluorophore-labeled IgE-Fc or Fcε3-4, and competitively displacing either donor fluorophore-labeled derCD23 by unlabeled sFcεRIα, or labeled sFcεRIα by unlabeled derCD23 (Fig. 8). It can be seen that sFcεRIα readily and completely displaces derCD23 from the complex with either IgE-Fc or Fcε3-4. Similarly, derCD23 can displace sFcεRIα almost completely from either IgE-Fc or Fcε3-4, but at very much higher concentrations as expected from the considerably lower binding affinity of

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derCD23. Positive controls for these FRET measurements were provided by displacement of labeled sFcεRIα by unlabeled sFcεRIα, and labeled derCD23 by unlabeled derCD23.

5 Discussion

The interaction between IgE and CD23 is critically involved in the allergic response at several stages, including allergen presentation, the regulation of IgE synthesis and transport of IgE and immune complexes across epithelial barriers in the gut and
10 airways. At the cell surface, mCD23 is trimeric, and sCD23 fragments shed from the membrane that contain sufficient stalk region are also trimeric, although the structure of the trimer has only been modeled based either upon the structures of other C-type lectins or guided by NMR chemical shift data. In the crystal structure of the complex reported here, two derCD23 “heads” bind to IgE, one to each heavy-
15 chain at a location between the Cε3 and Cε4 domains, and remote from the FcεRI binding site. The interaction is predominantly hydrophilic and dominated by salt bridges between positively charged CD23 residues and negatively charged IgE residues, despite the overall net positive charge (+9) of the Cε3 domain. The site on CD23, in agreement with that identified by NMR chemical shift mapping by titration of ¹⁵N-labeled derCD23 with monomeric Cε3 (titration with IgE-Fc led to the
20 formation of high molecular weight oligomers), is diametrically opposed to the connection to the α-helical coiled-coil stalk region (Fig. 5). This topology is such that an IgE molecule could not engage two heads from the same (modeled) CD23 trimer (as depicted in earlier cartoons), but could readily cross-link two mCD23 molecules. (The distance of 136Å between the Cα atoms of the N-terminal Phe158 residues of the
25 two derCD23 molecules, which are immediately adjacent to the top of the predicted α-helical coiled-coil stalk region, is such that it would require unraveling of about 30 residues, or almost one third of the stalk, to allow two heads from the same CD23 trimer to bind to the same IgE-Fc). Furthermore, the exposed location of the IgE-binding site on derCD23 strongly suggests that trimeric sCD23 could cross-link IgE
30 molecules either in solution or as mIgE on the cell surface, but a definitive statement must await determination of the structure of the CD23 trimer.

The binding of derCD23 in the cleft between the Cε3 and Cε4 domains fixes the angle between these two domains. Comparisons between Fcε3-4 and IgE-Fc structures
35 (the latter including Cε2 domains), both free and bound to sFcεRIα, show that while the (Cε4)₂ domain pair display no conformational variation and provide a fixed point

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of reference, the C ϵ 3 domains can adopt a range of “open” and “closed” conformations. The most open of these have been found in the complexes of Fc ϵ 3-4 and IgE-Fc with sFc ϵ RI α (Fig 11a-d) and occur in both ϵ -chains since this receptor engages with both C ϵ 3 domains (Fig. 7a). In contrast, CD23 binding causes the C ϵ 3 domains to adopt the most closed conformation that has yet been observed in any Fc ϵ 3-4 or IgE-Fc structure. This opening and closing of the C ϵ 3 domains in fact results from a flexing *within* the C ϵ 3 domain, an effect first noted in earlier structural comparisons. The AB loop/helix of C ϵ 3 interacts closely with C ϵ 4 (Fig. 6), and a change in the relative orientation of the EF loop/helix, which is contacted by derCD23 (Fig. 6), can clearly be seen when the Fc ϵ RI and CD23 complexes are compared (Fig. 7a). This intra-domain flexibility may be a result of the unique packing/stability profile of C ϵ 3 compared with all other C $_H$ domains, and in fact the isolated C ϵ 3 domain displays molten globule-like characteristics.

The consequence of these extreme open (Fc ϵ RI-bound) and closed (CD23-bound) conformations for the C ϵ 3 domains of IgE is that the binding of the two receptors are incompatible with each other (Figs. 7a-c). Clearly this is not, as previously thought, due to overlapping binding sites, but to a surprising allosteric linkage between the two distant sites. The idea that the open and closed conformations might interact differently with Fc ϵ RI and CD23 has been previously considered following a surprising discovery that CD23 bound more strongly to IgE at 4°C than 37°C, while the opposite was true for Fc ϵ RI. It was hypothesised that a change in the relative proportion of these open and closed conformational states might account for this temperature effect, and it will certainly be revealing to explore further the temperature dependence of the kinetics and thermodynamics of these receptor interactions as well as the conformational dynamics of the IgE molecule, particularly with respect to the C ϵ 3 domains.

IgE-Fc and IgE additionally contain the C ϵ 2 domains, and it is important to consider their effect upon the crystallographic results presented here for Fc ϵ 3-4. In IgE-Fc the (C ϵ 2)₂ domain pair packs asymmetrically against one of the C ϵ 3 domains in the bent IgE-Fc structure, and moves together with that C ϵ 3 domain when it opens up to accommodate sFc ϵ RI α binding. Modeling the (C ϵ 2)₂ domains onto the derCD23-Fc ϵ 3-4 complex shows that although there is no steric conflict that would prevent binding of either derCD23 molecule, the C ϵ 2 domains lie immediately adjacent to one

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of the derCD23 molecules and suggest that an interaction (stabilizing or destabilizing) might occur. This is consistent with the observed 2:1 stoichiometry for derCD23 binding to both Fc ϵ 3-4 and IgE-Fc. Although there is no evidence of any significant difference between the binding affinity of derCD23 for Fc ϵ 3-4, IgE-Fc or IgE, or between the binding of the two derCD23 molecules to IgE-Fc, a slightly faster on-rate for derCD23 binding to Fc ϵ 3-4 compared with both IgE-Fc and IgE has been reported. The biphasicity or dual affinity of CD23 binding to IgE that has been reported is a function of its oligomeric state and ability to engage IgE through more than one head, although as pointed out above, two heads of one CD23 almost certainly cannot engage the same IgE molecule and the avidity effect must result from trimeric CD23 binding to more than one IgE molecule.

The structural comparison of the derCD23 and sFc ϵ RI α complexes (Figs. 7b & c) also shows that sFc ϵ RI α binding prevents binding of *both* derCD23 molecules, and similarly that binding of *either* derCD23 molecule will prevent sFc ϵ RI α binding. The experimental data presented here (Fig. 8) confirm this observation. sFc ϵ RI α readily displaces derCD23 and, as expected, at approximately equimolar (5 μ M) concentrations of sFc ϵ RI α and IgE-Fc (or Fc ϵ 3-4) the displacement of derCD23 is virtually complete since this concentration of sFc ϵ RI α is very much greater than the $K_D \approx 1$ nM for sFc ϵ RI α binding. (The difference between the two curves in Fig.8a may reflect the slightly lower affinity reported for sFc ϵ RI α binding to Fc ϵ 3-4 compared with IgE-Fc). derCD23 can similarly cause almost complete displacement of sFc ϵ RI α , although much higher concentrations are required, greater than 100 μ M, in order to overcome its lower affinity ($K_D \approx 10^{-5} - 10^{-6}$ M).

CD23 belongs to the C-type (calcium-dependent) lectin superfamily and the presence of Ca²⁺ is known to enhance the affinity for IgE approximately 7-fold, although it is not essential for binding. A crystal structure of the head domain with a single bound Ca²⁺ ion has been solved, together with the Ca²⁺-free form, and an NMR structure also reports Ca²⁺ binding, but at an alternative site. However, no Ca²⁺ ions were observed in any of the six derCD23 molecules in the Fc ϵ 3-4 complex, despite the presence of 2 mM Ca²⁺ in the crystallization medium. In this regard, the existence of a disordered loop in derCD23 at the edge of the interface (residues 256-257 of loop 4, following earlier terminology) is intriguing. The crystal structure of the lectin head domain showed Ca²⁺ bound at a site involving residues Glu249 and Thr251 of loop 4.

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In that structure, residues 253-257 of loop 4 were also disordered, paradoxically becoming ordered in the *absence* of Ca^{2+} due to a rearrangement of the side-chain of Arg253, which occupied the Ca^{2+} site. The conformation of loop 4 seen in the complex differs from either the apo- or Ca^{2+} -bound structures; together with the adjacent loop 1, they represent the only main-chain conformational changes in derCD23 upon Fc ϵ 3-4 binding. The role of Ca^{2+} in IgE binding to CD23 thus remains unresolved at present, but it may involve reorganization of residues of the flexible loop 4 in the context of the complex, providing additional protein-protein interactions and thus enhancing affinity. There are precedents for the stabilizing effects of Ca^{2+} upon this and other loops in C-type lectin domains, such as mannose-binding protein in which carbohydrate binding is affected, and others where the binding of a protein partner is affected.

What is the functional significance of IgE's inability to engage both Fc ϵ RI and CD23 simultaneously? Although the affinity of derCD23 for IgE is considerably lower than that of Fc ϵ RI, avidity effects for trimeric CD23 can considerably enhance its ability to compete with Fc ϵ RI binding. Indeed, a recombinant sCD23 species trimerized *via* a leucine-zipper motif, termed lzCD23, more effectively inhibited IgE binding to mast cells expressing Fc ϵ RI. The mutual exclusion of Fc ϵ RI and CD23 binding to IgE is essential to prevent mast cell and basophil activation by trimeric sCD23, which could otherwise cross-link Fc ϵ RI-bound IgE on these cells. Similarly, it ensures that Fc ϵ RI cannot be cross-linked either by soluble IgE-CD23 complexes, or IgE bound to mCD23 (on B cells or APCs) in the absence of allergen. The mutual exclusion of Fc ϵ RI and CD23 binding is thus an important aspect of IgE biology, allowing it to function independently through its two receptors.

Furthermore, the location of the CD23 binding sites at points where the two heavy chains are most widely separated (in contrast to the Fc ϵ RI binding site where the chains approach each other most closely) and the connections to the stalk region (Fig. 5), maximize the propensity for cross-linking of mIgE on B cells committed to IgE synthesis by trimeric sCD23, and also mCD23 on B cells by IgE or IgE-allergen complexes. The inventors have hypothesized that through such interactions, the former leading to up-regulation of IgE synthesis and the latter to down-regulation, CD23 contributes to the mechanism of IgE homeostasis, and this notion has received experimental support from studies with monomeric and oligomeric sCD23 species. The structure of the complex is also consistent with the co-crosslinking of mCD21 and

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mIgE by trimeric sCD23 (proposed to enhance IgE up-regulation), since CD21 binds to the “tail” sequence that is, although only partially present in derCD23, located adjacent to the connection to the stalk [Fig. 5;]. However, the precise spatial arrangement of the IgE and CD21 binding sites on each derCD23 head and the relative positions of the heads in the CD23 trimer (as yet only modeled), together with the fact that two heads from one trimer almost certainly cannot engage a single IgE molecule, undoubtedly places topological constraints upon the formation of signaling complexes of these three molecules at the B cell surface.

The crystal structure of the derCD23-Fc ϵ 3-4 complex reported here provides the first structural basis for designing inhibitors with the potential to interfere with IgE regulation and other CD23-mediated processes. Of wider significance to IgE and allergic disease, the structure also reveals the mechanism for an allosteric connection between the distantly located binding sites for IgE’s two principal receptors, and demonstrates that allosteric inhibition is a viable strategy to target either of these receptor interactions for therapeutic purposes.

Summary

The role of immunoglobulin E (IgE) in allergic disease mechanisms is performed principally through its interactions with two receptors, Fc ϵ RI on mast cells and basophils, and CD23 (Fc ϵ RII) on B cells. The former mediates allergic hypersensitivity, the latter regulates IgE levels, and both receptors, also expressed on antigen presenting cells, contribute to allergen uptake and presentation to the immune system. The inventors have solved the crystal structure of the soluble lectin-like “head” domain of CD23 (derCD23) bound to a sub-fragment of IgE-Fc consisting of the dimer of C ϵ 3 and C ϵ 4 domains (Fc ϵ 3-4). One CD23 head binds to each heavy chain at the interface between the two domains, explaining the known 2:1 stoichiometry and suggesting mechanisms for cross-linking membrane-bound trimeric CD23 by IgE, or membrane IgE by soluble trimeric forms of CD23, both of which may contribute to the regulation of IgE synthesis by B cells. The two symmetrically located binding sites are distant from the single Fc ϵ RI binding site, which lies at the opposite ends of the C ϵ 3 domains. Structural comparisons with both free IgE-Fc and its Fc ϵ RI complex reveal not only that the conformational changes in IgE-Fc required for CD23 binding are incompatible with Fc ϵ RI binding, but also that the converse is true. The two binding sites are allosterically linked. The inventors demonstrate experimentally the reciprocal inhibition of CD23 and Fc ϵ RI binding in solution, and suggest that the mutual exclusion of receptor binding allows IgE to function independently through its two receptors.

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Claims

1. A variant IgE Fc region comprising at least one amino acid modification in the C ϵ 3 domain relative to a wild-type IgE Fc region, wherein the at least one amino acid in the wild-type IgE Fc region which is modified is part of a CD23 binding site, and
5 wherein the variant IgE Fc region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc region.
2. A variant IgE Fc region according to claim 1, wherein the variant IgE Fc region exhibits ablated binding to CD23 (Fc ϵ RII), but is capable of binding to Fc ϵ RI.
10
3. A variant IgE Fc region according to either claim 1 or 2, wherein the variant IgE Fc region is derived from the wild-type human IgE Fc comprising an amino acid sequence substantially as set out in SEQ ID No:2, or a functional variant or fragment thereof.
15
4. A variant IgE Fc region according to any preceding claim, wherein the variant IgE Fc region is derived from the wild-type human IgE Fc region which is encoded by a nucleic acid sequence substantially as set out in SEQ ID No:1, or a functional variant or fragment thereof.
20
5. A variant IgE Fc region according to any preceding claim, wherein the at least one amino acid which is modified is any one of amino acid residues 329-441 of SEQ ID No:2.
- 25 6. A variant IgE Fc region according to any preceding claim, wherein the at least one amino acid modification in the variant IgE Fc region is not in the A-B helix of the C ϵ 3 domain of IgE.
7. A variant IgE Fc region according to any preceding claim, wherein the variant
30 IgE Fc region comprises at least one amino acid modification in the E-F helix of the C ϵ 3 domain of IgE, in the C-D loop of the C ϵ 3 domain of IgE and/or in the C-terminal region of the C ϵ 3 domain of IgE.
8. A variant IgE Fc region according to any preceding claim, wherein the variant
35 IgE Fc region comprises at least one amino acid modification at a position corresponding to wild-type IgE Fc region selected from the group consisting of: (i)

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amino acids 404-415 from the E-F helix of IgE; (ii) amino acids 376-380 from the C-D loop of IgE; and (iii) residue 433-441 from the C-terminal region of IgE.

9. A variant IgE Fc region according to any preceding claim, wherein the variant
5 IgE region comprises at least one amino acid modification at a position
corresponding to wild-type IgE Fc region selected from the group consisting of: 376;
377; 378; 379; 380; 404; 405; 406; 407; 408; 409; 410; 411; 412; 413; 414; 415; 433;
434; 435; 436; 437; 438; 439; 440; and 441 of SEQ ID No:2, and preferably at
position 376; 378; 381; 408; 409; 411; 412; 435; 439; and/or 440 of SEQ ID No:2.
10. A variant IgE Fc region according to any preceding claim, wherein the variant
IgE region comprises at least one amino acid modification at a position equivalent to
409, 412, 376, 380 and/or 435 of the wild-type human IgE Fc region.
11. A variant IgE Fc region according to any preceding claim, wherein the at least
15 one modification comprises an amino acid substitution.
12. A variant IgE Fc region according to any preceding claim, wherein the at least
one modification comprises a non-conservative substitution.
13. A variant IgE Fc region according to any preceding claim, wherein the variant
Fc region comprises at least one amino acid modification selected from: D409A;
E412A; R376A; K380A and K435A.
14. A variant IgE Fc region according to any preceding claim, wherein the variant
25 human IgE Fc region comprises more than one modification in the C ϵ 3 domain of the
Fc region equivalent to the human IgE Fc region of a wild-type antibody.
15. A variant IgE Fc region according to any preceding claim, wherein the variant
30 human IgE Fc region comprises at least two, three, four or five modifications at any
of the amino acids at positions equivalent to 376; 377; 378; 379; 380; 404; 405; 406;
407; 408; 409; 410; 411; 412; 413; 414; 415; 433; 434; 435; 436; 437; 438; 439; 440;
and 441 of wild-type human IgE-Fc.
16. A variant IgE Fc region according to any preceding claim, wherein the variant
35 Fc region comprises a modification at each of the amino acids at positions equivalent
to 409, 412, 376, 380 and/or 435 of wild-type human IgE-Fc.

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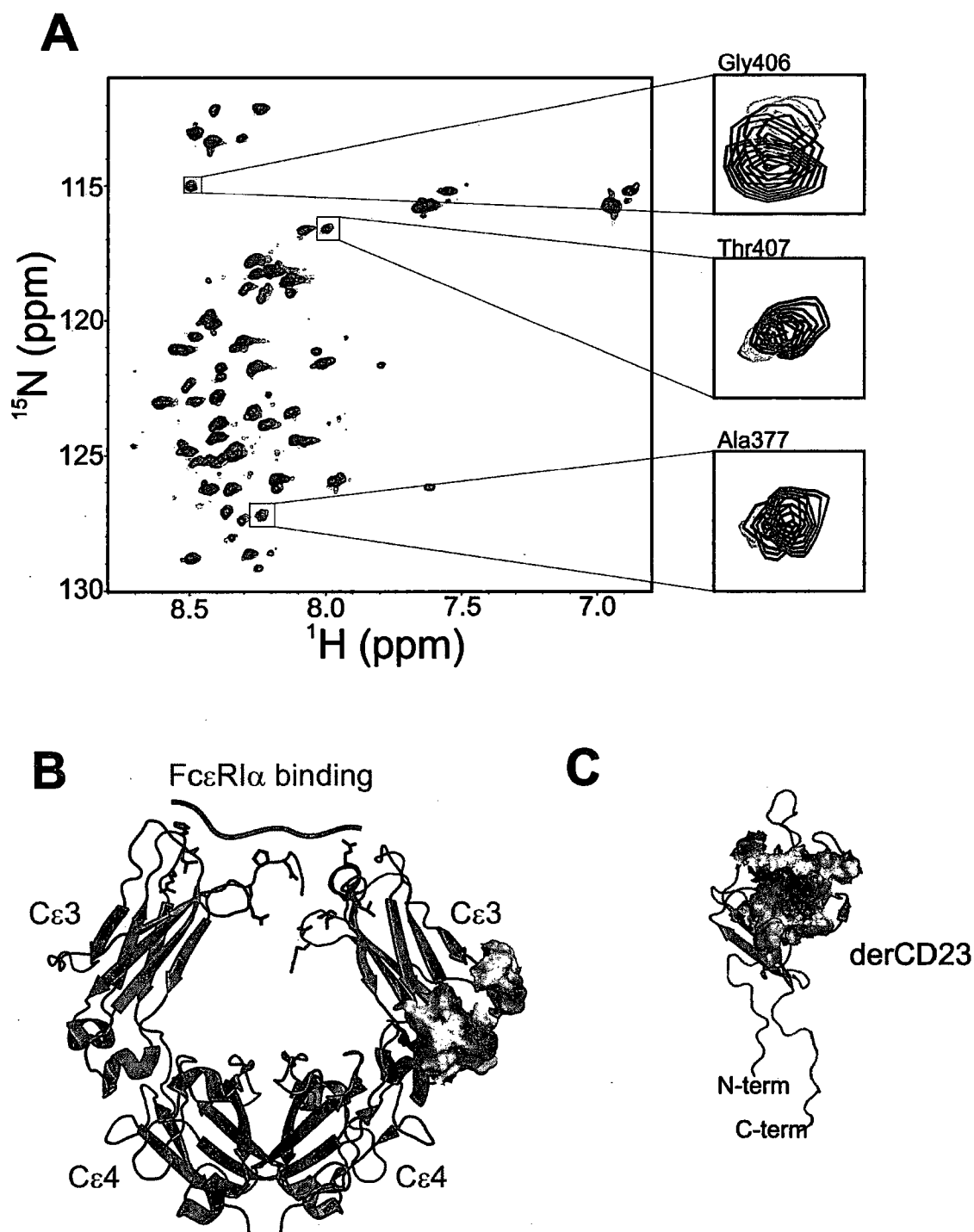
17. A variant IgE Fc region according to any preceding claim, wherein the variant IgE Fc region comprises an amino acid sequence substantially as set out in any one of SEQ ID No: 3-7, or a functional variant or fragment thereof.
- 5 18. A variant IgE Fc region according to any preceding claim, wherein the equilibrium constant of dissociation (K_D or K_d) of the variant IgE Fc region for CD23 is at least $5\mu\text{M}$, $10\mu\text{M}$, $25\mu\text{M}$ or greater.
- 10 19. A variant IgE Fc region according to any preceding claim, wherein the variant IgE Fc region is capable of binding to FcεRI with a K_A value of at least 10^7 M^{-1} .
20. A nucleic acid encoding the variant IgE Fc region according to any one of claims 1-19.
- 15 21. A nucleic acid according to claim 20, wherein the nucleic acid comprises a nucleotide sequence substantially as set out in any one of SEQ ID No: 8-12, or a functional variant or fragment thereof.
- 20 22. A genetic construct comprising the nucleic acid according to either claim 20 or claim 21.
23. A recombinant vector comprising the genetic construct according to claim 22.
- 25 24. A host cell comprising the genetic construct according to either claim 20 or claim 21, or the recombinant vector according to claim 23.
- 30 25. A method of preparing a variant IgE Fc region, the method comprising- (i) culturing at least one cell according to claim 24 under conditions suitable for the expression of a variant IgE Fc region; and (ii) isolating the variant IgE Fc region.
- 35 26. A method of producing a variant IgE Fc region which exhibits a reduced binding affinity for CD23, the method comprising modifying at least one amino acid in the Cε3 domain of a wild-type IgE Fc region, wherein the at least one amino acid in the wild-type IgE Fc region which is modified is part of the CD23 binding site, and wherein the variant IgE Fc region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc region.

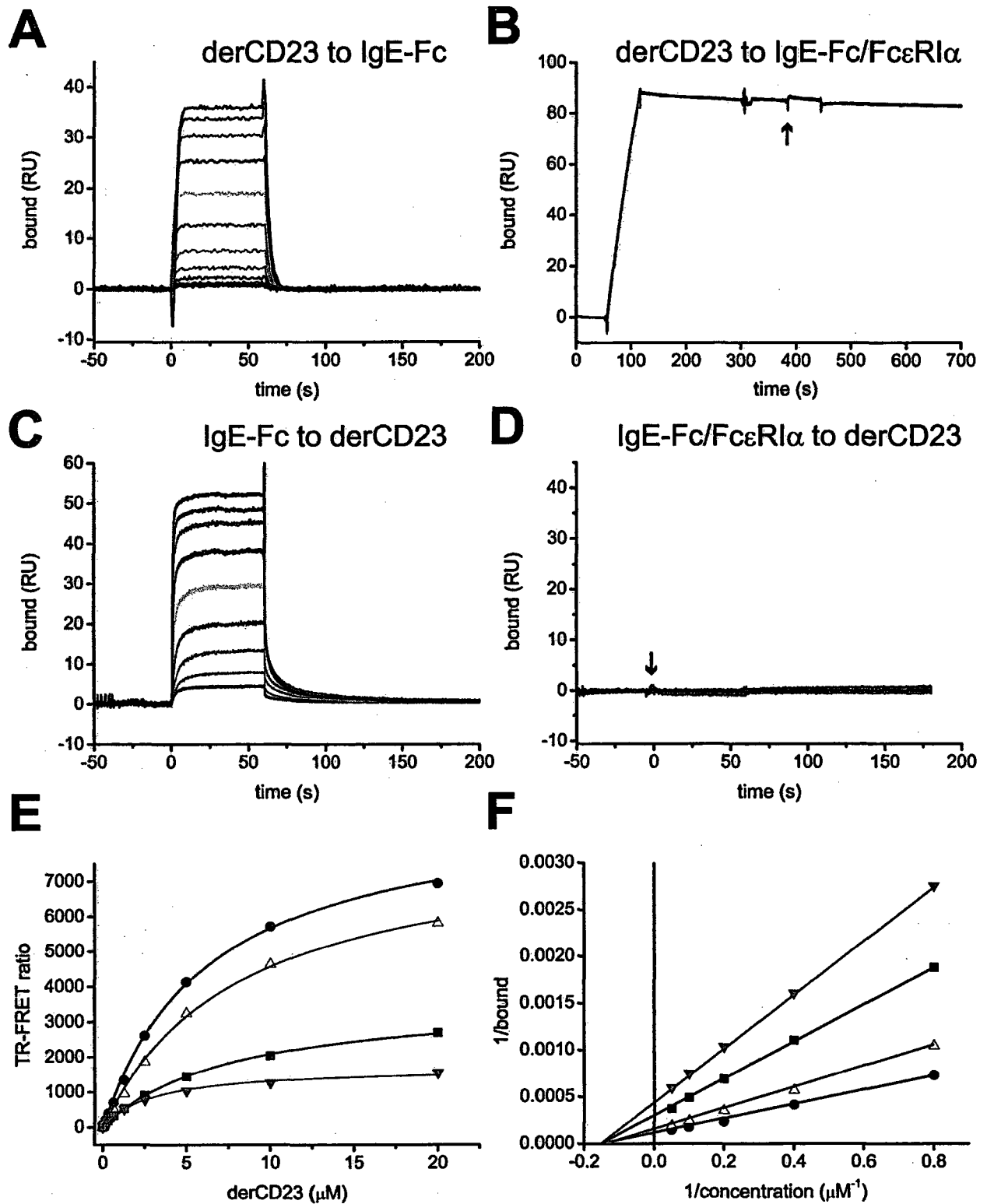
- 53 -

27. A method according to claim 26, wherein the variant IgE Fc region produced by the method is as defined in any one of claims 1-19.
- 5 28. A method according to either claim 25 or claim 26, wherein the wild-type IgE Fc region which is modified is part of a therapeutically active IgE antibody.
29. A method according to either claim 25 or claim 26, wherein the method comprises initially creating the variant IgE Fc region and then fusing it to a
10 functional fragment of an IgE antibody to produce an Fc fusion, for example a chimera.
30. An antibody or a functional fragment thereof comprising the variant IgE Fc region of any one of claims 1-19.
- 15 31. An antibody or a functional fragment thereof according to claim 30, wherein the antibody or functional fragment thereof is incapable of binding to CD23 (FcεRII), or exhibits a reduced binding affinity thereto, but is capable of binding to FcεRI.
- 20 32. An antibody or a functional fragment thereof according to either claim 30 or claim 31, wherein the antibody or functional fragment thereof is monoclonal or polyclonal.
33. An antibody or a functional fragment thereof according to any one of claims
25 30-32, wherein the functional fragment comprises any of the fragments selected from a group consisting of VH, VL, Fd, Fv, Fab, Fab', scFv, F(ab')₂ and Fc fragment.
34. A variant IgE Fc region according to any one of claims 1-19, or an antibody or a functional fragment thereof according to any one of claims 30-33, for use in therapy
30 or diagnosis.
35. A variant IgE Fc region according to any one of claims 1-19, or an antibody or a functional fragment thereof according to any one of claims 30-33, for use in treating, preventing or ameliorating a CD23-mediated side effect.
- 35

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36. A variant IgE Fc region, or an antibody or a functional fragment thereof according to claim 35, wherein the CD23-mediated side effect is prevented in IgE antibody treatment or therapy.
- 5 37. A variant IgE Fc region, or an antibody or a functional fragment thereof according to either claim 35 or 36, wherein the CD23-mediated side effect is prevented during the treatment of cancer, autoimmune disease or infectious disease.
- 10 38. A variant IgE Fc region, or an antibody or a functional fragment thereof according to any one of claims 35-37, wherein the CD23-mediated side effect is CD23-dependent epitope spreading.
- 15 39. A pharmaceutical composition comprising a variant IgE Fc region according to any one of claims 1-19, or an antibody or a functional fragment thereof according to any one of claims 30-33; and optionally a pharmaceutically acceptable vehicle.
- 20 40. A process for making the composition according to claim 39, the process comprising combining a therapeutically effective amount of a variant IgE Fc region according to any one of claims 1-19, or an antibody or a functional fragment thereof according to any one of claims 30-33, with a pharmaceutically acceptable vehicle.

1/12**Figure 1**

2/12**Figure 2**

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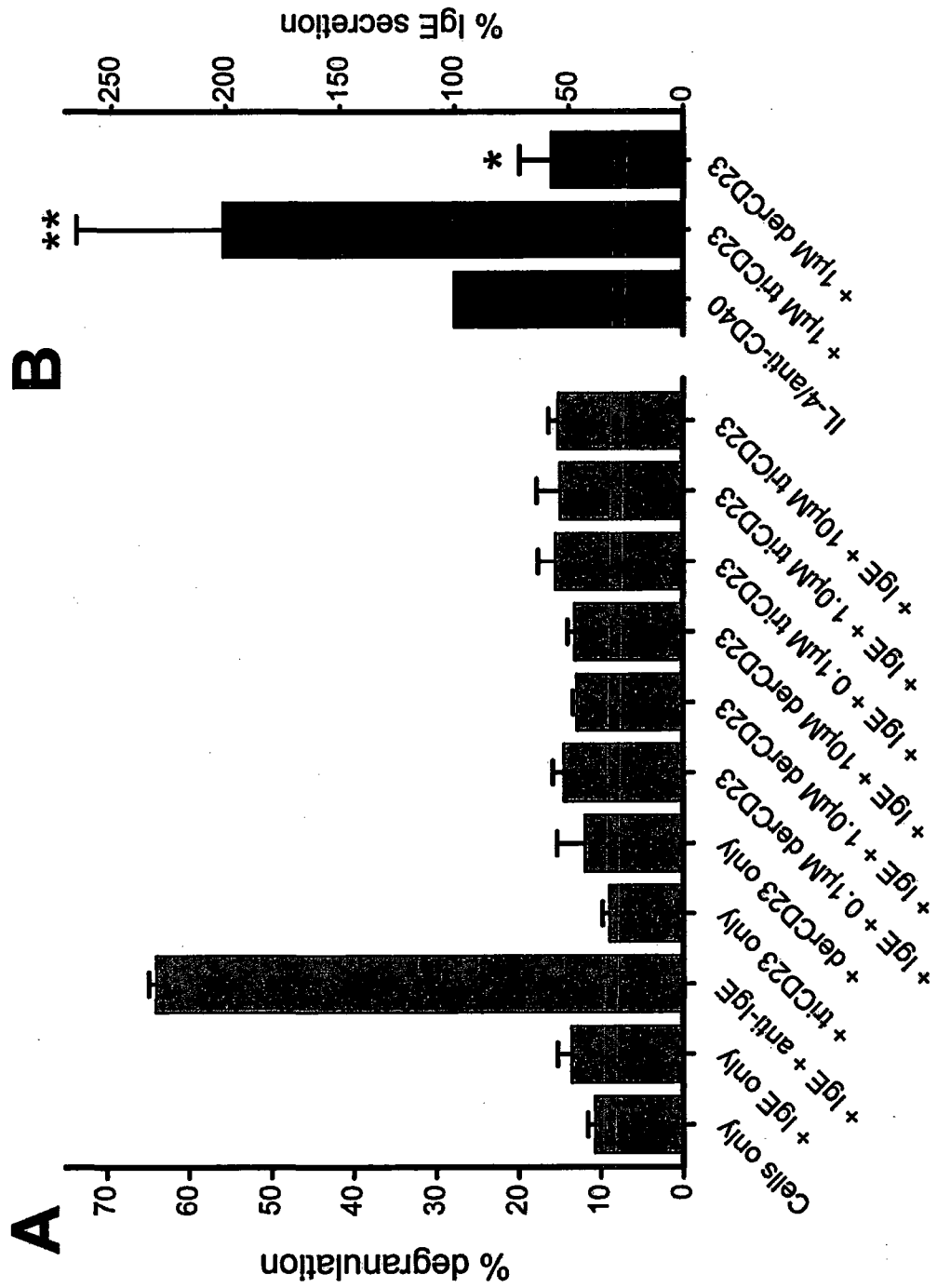
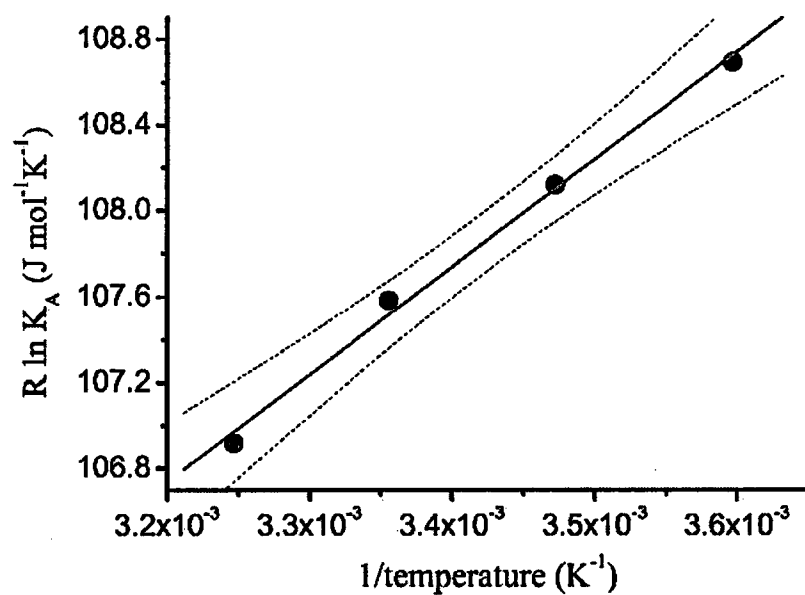
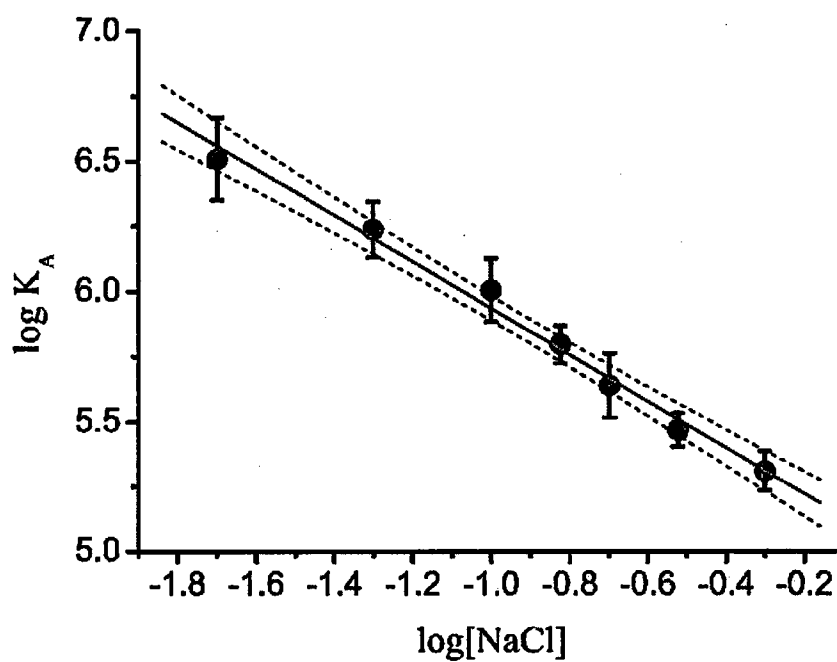
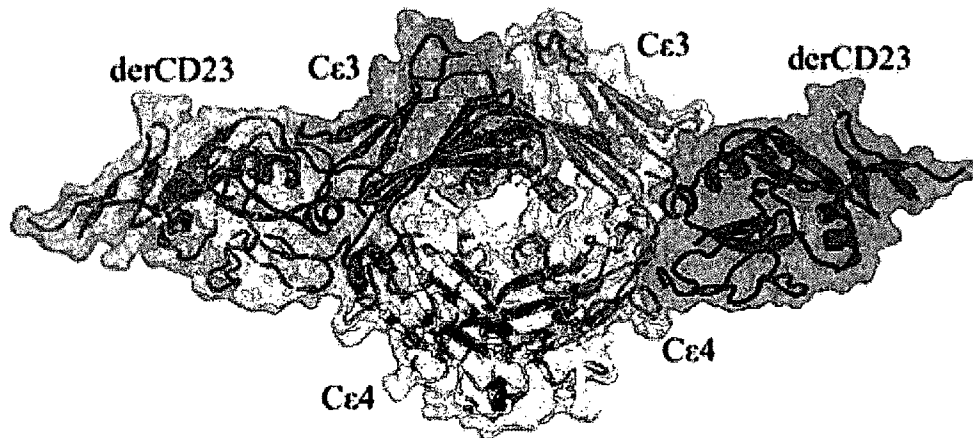
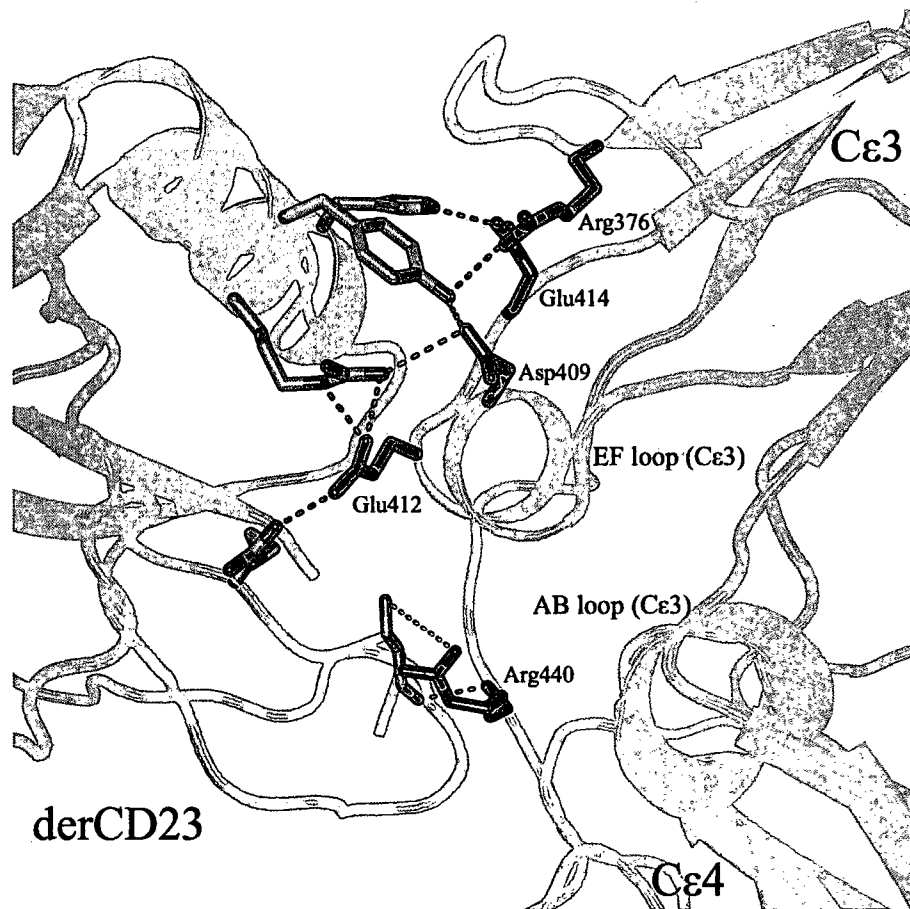


Figure 3

4/12**Figure 4****A****B**

5/12**Figure 5****Figure 6**

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Figure 7a

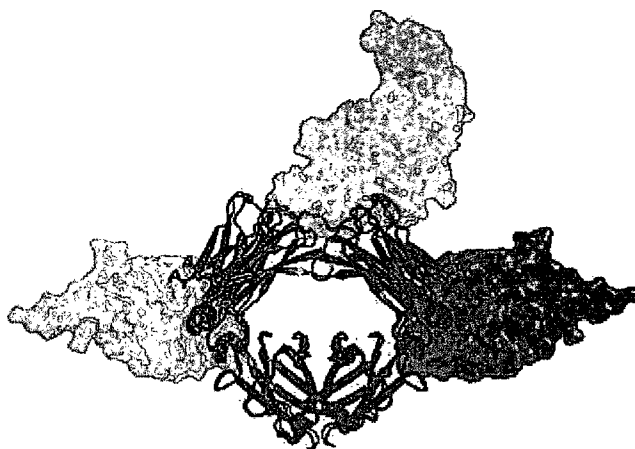
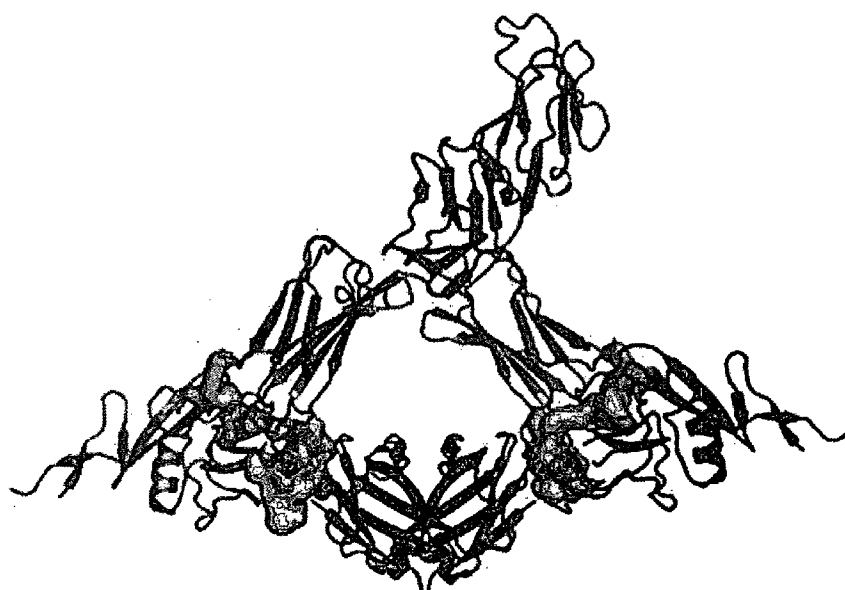
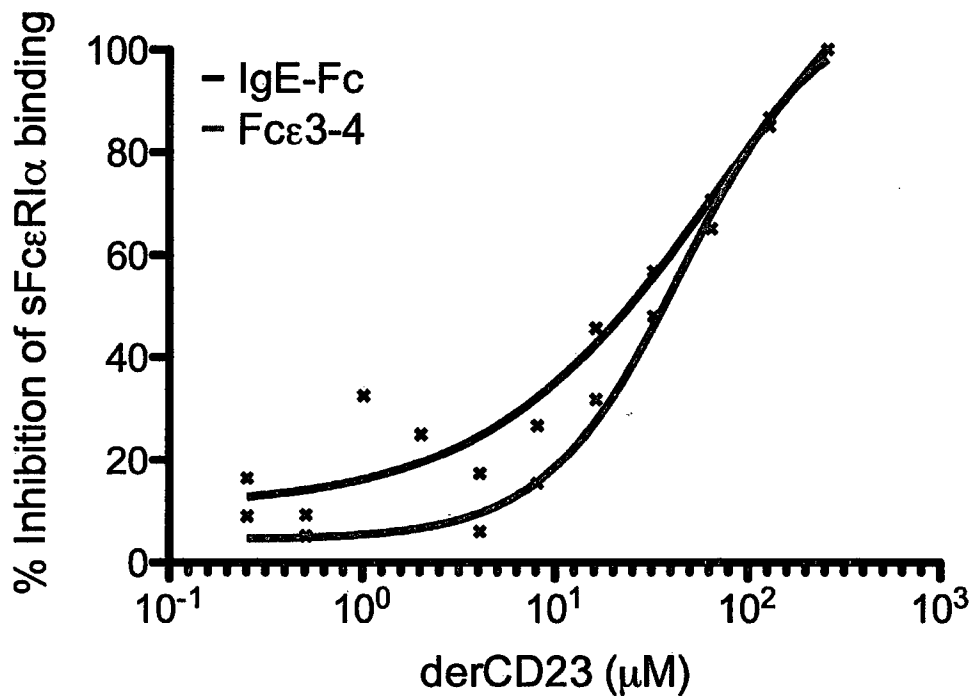
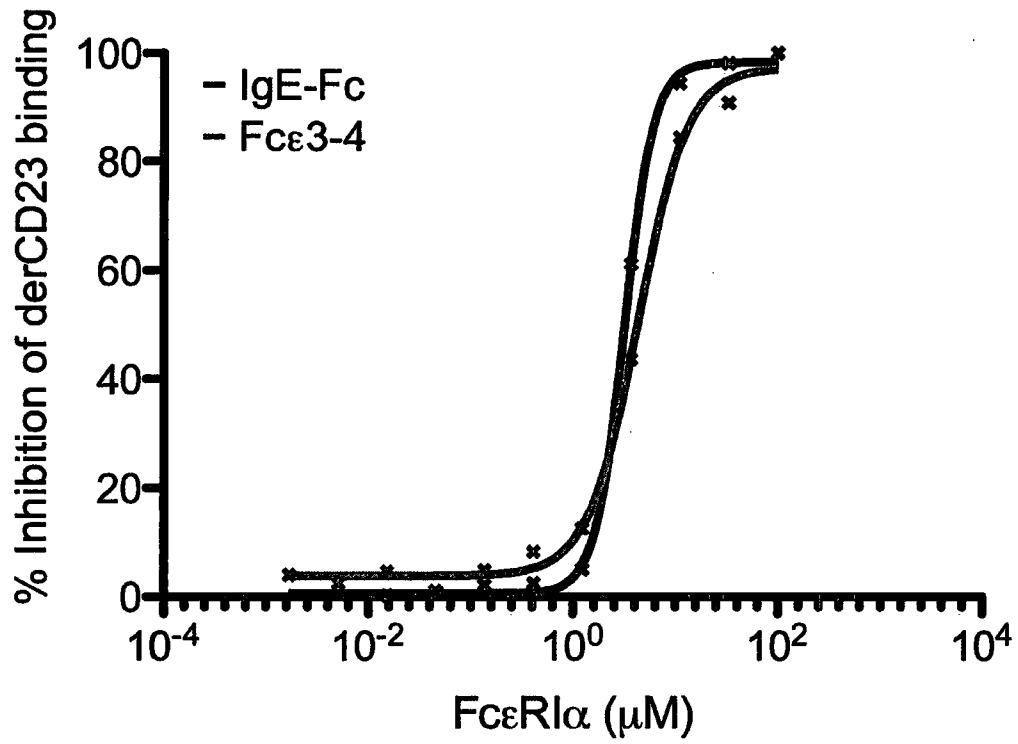


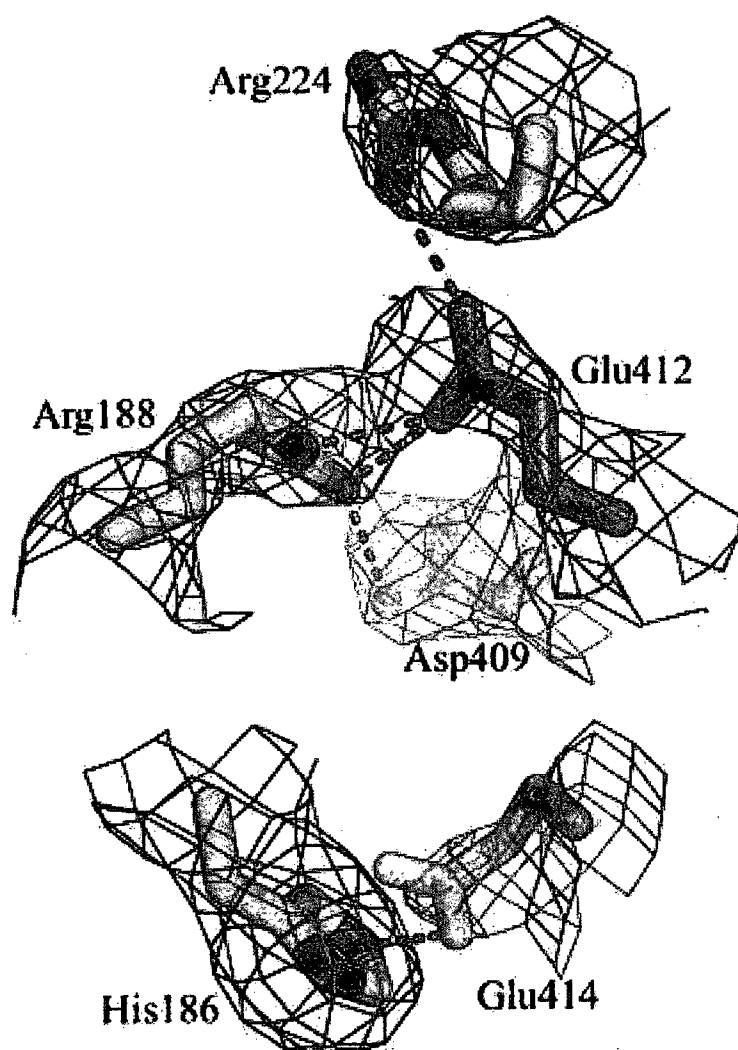
Figure 7b



Figure 7c



7/12**Figure 8**

8/12Figure 9

9/12

Figure 10a

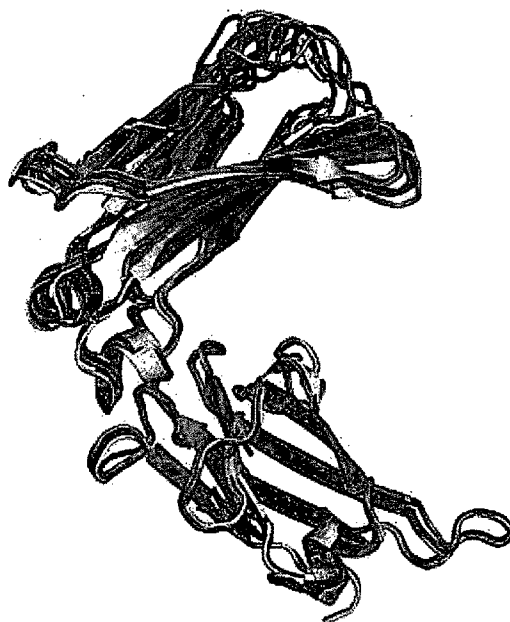
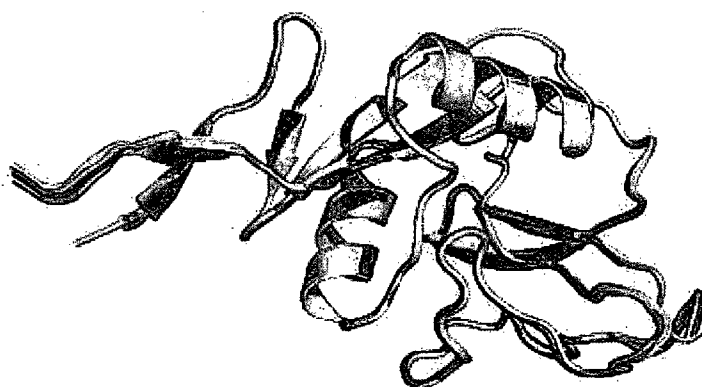


Figure 10b



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Figure 11a

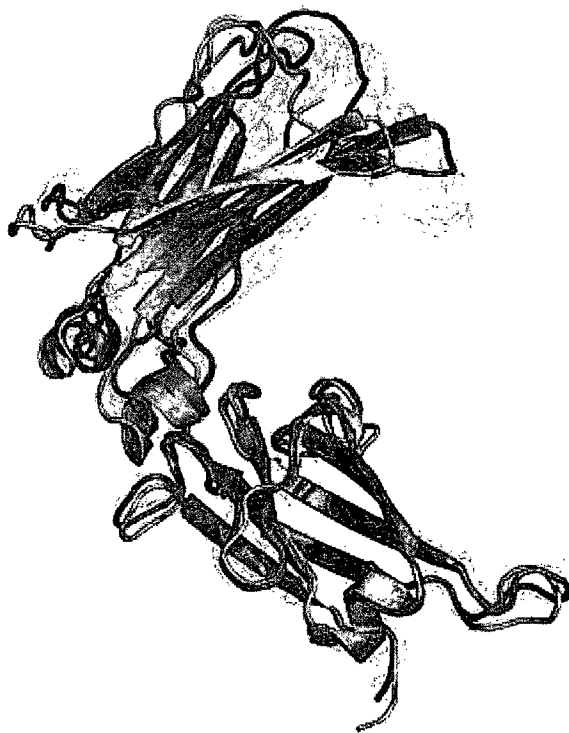
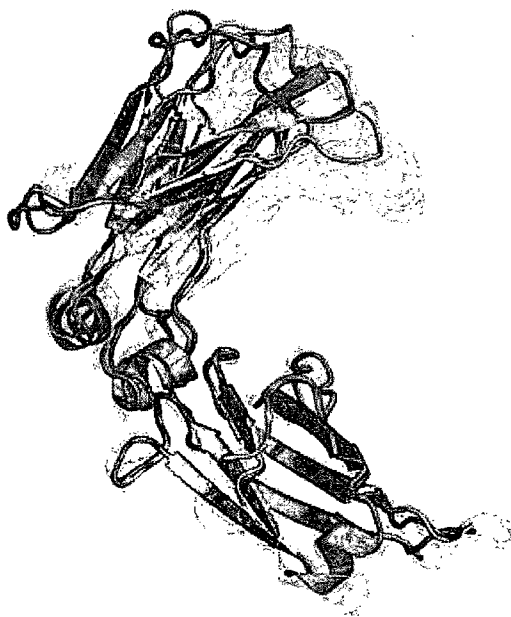


Figure 11b



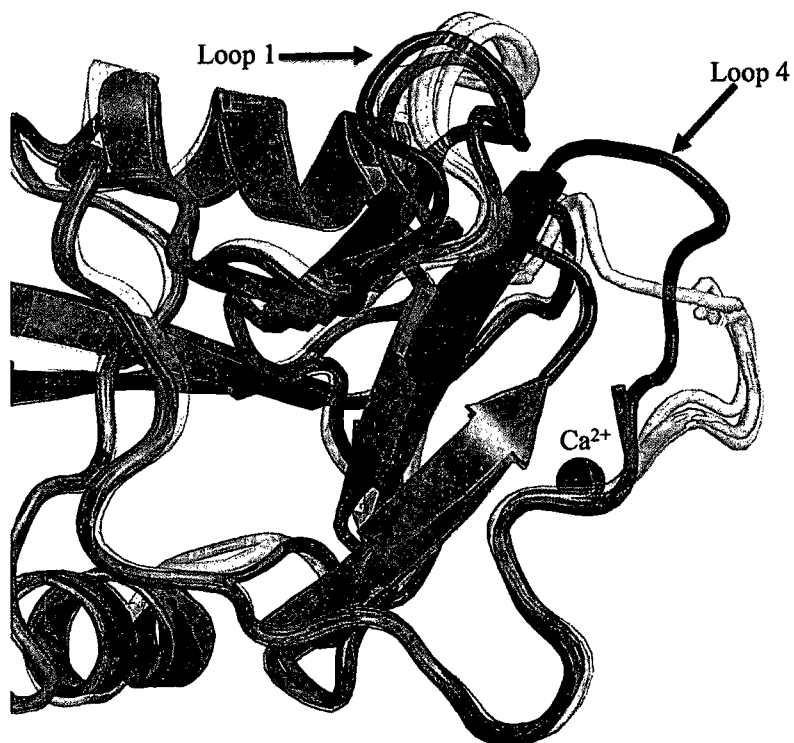
11/12

Figure 11c



Figure 11d



12/12**Figure 12**

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2013/051804

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K16/00 C07K16/46 A61P37/08
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/04173 A1 (GENENTECH INC [US]) 4 March 1993 (1993-03-04) tables 6-7, 11 page 40 - page 43 -----	1-34, 39, 40

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

7 August 2013

Date of mailing of the international search report

23/10/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Bumb, Peter

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2013/051804

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
☐ on paper
☐ in electronic form
 - b. (time)
☐ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2013/051804

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-34, 39, 40(all partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-34, 39, 40(all partially)

A variant IgE Fc-region comprising at least one amino acid modification in the C γ 3-domain relative to a wild-type IgE Fc region, wherein the at least one amino acid in the wild-type IgE Fc region which is modified is part of a CD23 binding site and wherein the variant IgE Fc-region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc-region,
wherein the modification is at position 376 of SEQ ID No: 2.

2. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 377.

3. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 378.

4. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 379.

5. claims: 1-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 380.

6. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 404.

7. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 405.

8. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 406.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

9. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)
same as for invention 1, excepts that the modification is at
position 407.

10. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all
partially)
same as for invention 1, excepts that the modification is at
position 408.

11. claims: 1-34, 39, 40(all partially)
same as for invention 1, excepts that the modification is at
position 409.

12. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all
partially)
same as for invention 1, excepts that the modification is at
position 410.

13. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all
partially)
same as for invention 1, excepts that the modification is at
position 411.

14. claims: 1-34, 39, 40(all partially)
same as for invention 1, excepts that the modification is at
position 412.

15. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all
partially)
same as for invention 1, excepts that the modification is at
position 413.

16. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all
partially)
same as for invention 1, excepts that the modification is at
position 414.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

17. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 415.

18. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 433.

19. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 434.

20. claims: 1-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 435.

21. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 436.

22. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 437.

23. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 438.

24. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 439.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

25. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 440.

26. claims: 1-9, 11, 12, 14, 15, 18-20, 22-34, 39, 40(all partially)

same as for invention 1, excepts that the modification is at position 441.

27. claims: 35-38

A variant IgE Fc-region comprising at least one amino acid modification in the C?3-domain relative to a wild-type IgE Fc region, wherein the at least one amino acid in the wild-type IgE Fc region which is modified is part of a CD23 binding site and wherein the variant IgE Fc-region exhibits a reduced binding affinity to CD23 compared to the wild-type IgE Fc-region,
for use in treating, preventing or ameliorating a CD23-mediated side-effect.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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