HUMANIZED ANTI-IgE ANTIBODIES THAT CROSSLINK CD23 ON B LYMPHOCYTES BUT DO NOT SENSITIZE MAST CELLS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. provisional application no. 62/013196, filed June 17, 2014, the entireties of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] 1. FIELD OF THE INVENTION

[0003] The present invention pertains to the preparation of humanized antibodies, which are capable of binding to free IgE, membrane-bound IgE on B lymphocytes, IgE bound by CD23, but not to IgE bound by high-affinity IgE.Fc receptors on mast cells. The invention also pertains to the therapeutic applications of such antibodies in treating IgE-mediated diseases, including allergic asthma, allergic rhinitis, atopic dermatitis, food allergy, chronic spontaneous (idiopathic) urticaria, chronic rhinosinusitis, systemic mastocytosis, cutaneous mastocytosis, allergic bronchopulmonary aspergillosis, recurrent idiopathic angioedema, and eosinophil-associated gastrointestinal disorder.

[0004] 2. DESCRIPTION OF RELATED ART

[0005] Allergy is a hypersensitive state induced by an exaggerated immune response to a foreign, harmless antigen. An immediate hypersensitivity response occurs through the interaction of immunoglobulin E (IgE) and the high-affinity IgE.Fc receptor (FcεRI) present on the surface of mast cells and basophils in the presence of allergen which is capable of reacting with the FcεRI-bound IgE, causing the release of performed

and newly-synthesized mediators from these inflammatory cells. Most allergic diseases are IgE-mediated. It is well known that a number of non-allergic diseases, which do not involve an immune response to a foreign antigen, particularly inflammatory skin diseases, are also IgE-mediated.

[0006] There are two major receptor for IgE, FcεRI and the low-affinity IgE.Fc receptor FcεRII (also referred to as CD23). FcεRI is predominantly expressed on the surface of mast cells and basophils in humans, where it is a tetrameric complex consisting of one α-chain, one β-chain, and two disulfide-bound γ-chains. Activation of FcεRI on mast cells and basophils by allergens leads to degranulation, while activation of FcεRI on dendritic cells leads to IgE-mediated allergen presentation.

[0007] CD23 is a type II transmembrane glycoprotein of approximately 45kDa molecular weight comprising a C-type lectin-like domain, followed by a stalk region, which bears several repeats that serve as a putative leucine zipper to form coiled-coil trimers, a single membrane-spanning region, and a short N-terminal cytoplasmic domain. Indeed, the affinity of monomeric CD23 to IgE ($KD=10^{-6}$-10^{-7} M) is much lower than that of FcεRI (KD= about 10^{-10} M), but substantially increased in its trimeric form ($KD=10^{-8}$-10^{-9} M). The FCER2 gene encoding CD23 polypeptide possesses 2 alternative transcription start sites driving synthesis of two mRNA variants which leads to produce two CD23 isoforms differing in the first seven (CD23a) and six (CD23b) amino acids of the N-terminal cytoplasmic tail. CD23a is exclusively and contiguously expressed by B cells, whereas CD23b is induced by IL-4 on the surface of monocyte/macrophage, B cells, T cells, eosinophils, dendritic cells, and epithelial cells. CD23 on B cells is thought to contribute to regulation of IgE production and antigen presentation, while CD23 on macrophages

and dendritic cell is involved in phagocytosis/endocytosis, clearance of antigen-IgE complex and antigen presentation. Additional function of CD23 on epithelial cells includes the transportation of IgE. The antigen-IgE complex directly penetrate across the epithelium to the lumen space and vice versa. CD23 can also be released from cell surfaces as a range of free soluble CD23 (sCD23) proteins of 37, 33, 25, and 16 kDa. The predominant metalloprotease responsible for CD23 shedding in vivo is ADAM10 gene, which generates 37 kDa or 35kDa sCD23 species in a trimeric form. A further naturally occurring sCD23 is derCD23 produced by action of the Der p1 protease found in the faeces of the house dust mite, *dermatophagoides pteronyssinus*. The cleavage of CD23 by Der p1 yields the 16 kDa monomeric derCD23. It is shown that trimeric sCD23 fragments were observed to be key molecules for potentiating spontaneous IgE synthesis, whereas the smaller monomeric sCD23 appears to down-regulate IL-4 stimulated IgE synthesis.

[0008] IgE exists in a secreted form and a membrane-bound form, which appears to be splicing variants. The constant regions of the ε chain of secreted form of IgE harbors C_{H1}-C_{H2}-C_{H3}-C_{H4} domains, whereas the ε chain of membrane-bound form of IgE having a membrane-bound IgE (mIgE) is found in two isoforms as a result of alternative splicing in humans. The ε chain of both isoforms of human mIgE contains a migis-ε and a membrane-anchoring peptide. One isoform contains only the migis-ε peptide (15 amino acid long) between the CH4 domain and the membrane-anchoring peptide (referred to as "short form"), whereas the second isoform additionally contains an extra 52 amino acid long domain (referred to as CεmX domain) between the C_{H4} domain and the migis-ε peptide (referred to as "long form").

[0009] Since IgE plays a central role in mediating most allergic diseases, several strategies have been proposed to control IgE levels in the body or to regulate IgE synthesis, such as anti-IgE, anti-IL-4/IL-13 and anti-CD23. Omalizumab (Xolair®) is a recombinant humanized monoclonal anti-IgE antibody that binds to free IgE circulating in the serum and membrane-bound IgE on B cells but not FcεRI-bound and CD23-bound IgE on cell surface. Omalizumab causes significant (up to 99%) reduction of serum free IgE in allergic patients, leading to inhibit IgE binding to FcεRI and subsequently a down-regulation of FcεRI on basophils and mast cells. Numerous clinical trials in many IgE-mediated diseases, such as allergic asthma, chronic urticaria, allergic rhinitis, atopic dermatitis, etc., have shown that Omalizumab is efficacious and safe in treating these diseases. Omalizumab has been approved for treating severe allergic asthma and chronic idiopathic urticaria in many countries. Moreover, lumiliximab, which is an anti-CD23 monoclonal antibody consisting of primate (cynomolgus macaque) variable regions and human constant regions, is found to bind to the C-type lectin domain preventing IgE from binding to CD23, thus leading to stabilization of surface CD23 and reduction of the proteolytic cleavage of CD23. Lumiliximab has been reported to inhibit the germline ε transcripts, decrease the IgE production of human peripheral blood mononuclear cell (PBMCs) in culture, and reduce blood IgE levels in allergic patients.

[0010] In a 1990 patent (US Pat. No. 4,940,782), a murine IgG monoclonal antibody 44.7b specific for rat IgE, which is capable of binding to CD23-bound IgE but not IgE bound to FcεRI, was disclosed. This antibody 44.7b was never pursued as a therapeutic candidate for treating IgE-mediated diseases. A paper published in 2012 (Shiung et al., *Immunobiology*, 2012, 217:676-683) reported the discovery of a murine

anti-human IgE monoclonal antibody, 8D6, which is capable of binding to CD23-bound IgE but not IgE bound to FcεRI. The authors suggested that this antibody possesses not only the major pharmacologic properties of Omalizumab to neutralize IgE without sensitizing mast cells and basophils but also the property of lumiliximab through crosslinking IgE bound to CD23. However, the murine antibody may be immunogenic and cannot be applied for treating IgE-mediated diseases. The murine antibody for therapeutic applications in man has been found to be limited by immune responses made by patients to the murine antibodies. Thus, the humanization of murine antibodies is necessary to reduce immunogenicity in human recipients.

SUMMARY

[0011] The present invention pertains to humanized antibodies that bind to (1) human IgE in its secreted free form, (2) to membrane-bound IgE on B lymphocytes, and (3) to IgE bound by CD23 on B cells and by free CD23 in solution. Such anti-IgE antibodies do not bind to IgE bound by FcεRI on mast cells and basophils and hence do not activate those inflammatory cells. The present invention also pertains to the biological properties of those humanized anti-IgE antibodies in the biosynthesis of IgE, the binding of IgE to various cellular and molecular components, and the potential clearing effects of allergens. It also pertains to the application of such antibodies in the treatment of IgE-mediated allergic and non-allergic diseases.

[0012] We have recognized that the latest results of the phase Ib and IIa trials of the anti-M1' antibody, MEMP1972A (Gauvreau et al., *Science Translation Medicine*, 2014, Vol. 6: 243ra85), suggested that by inhibiting new IgE synthesis, IgE-mediated symptoms can be decreased. We also conceptualize that since hu8D6:IgE complexes can bind to

CD23 and since CD23 is expressed in numerous cell types (Acharya et al. Clin. Exp. Immunol., 2010, 162:12-23), the absorption of hu8D6:IgE complexes to cell surface CD23 can reduce the total IgE levels (free and hu8D6-bound IgE) in circulation in vivo. Besides, epithelial cells along the mucosal lining are capable of clearing cell-bound substances through CD23-dependent transcytosis (Tu et al. Gastroenterology, 2005, 129:928-940), hu8D6 may facilitate the mopping up and removal of incoming allergens and endogenous IgE-binding autoantigens when hu8D6:IgE complexes are transported into the lumen space of gastrointestinal and bronchoalveolar tracts. We also rationalize that the binding of hu8D6 to CD23-bound IgE can effectively crosslink CD23 on B lymphocytes to result in inhibition of antigen-specific IgE production by B cells upon activation by a specific antigen. Those properties of hu8D6 should support the potential utility that hu8D6 and other humanized antibodies with the same set of IgE-binding specificities can inhibit antigen-specific IgE production in patients with IgE-mediated diseases and hence ameliorate those diseases. Using murine 8D6 antibody as an example, we have prepared a humanized form of the antibody. This humanized antibody possesses all binding and biological properties discussed above.

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BRIEF DESCRIPTION OF THE DRAWINGS

[0013] The present description will be better understood from the following detailed description read in light of the accompanying drawings, where:

[0014] Figure **1** illustrates the alignment of amino acid sequences of variable heavy chains including human germline V_H1-69 and JH4 sequences (V_H1-69/JH4), murine anti-IgE antibody 8D6 (mu8D6), and 8D6-grafted humanized 8D6 (hu8D6). Complementarity determining regions (CDRs) are underlined. The amino acids of murine V_H framework retained in hu8D6 are labeled by star.

[0015] **Figure 2** illustrates the alignment of amino acid sequences of variable light chains including human germline Vκ1-39 and Jκ1 sequences (Vκ1-39/Jκ1), murine anti-IgE antibody 8D6 (mu8D6), and 8D6-grafted humanized 8D6 (hu8D6). Complementarity determining regions (CDRs) are underlined. The amino acids of murine Vκ framework retained in hu8D6 are labeled by star.

[0016] **Figure 3** illustrates the titration curves of hu8D6 (solid line) and the parent c8D6 antibody (dot line) binding to human IgE by ELISA.

[0017] **Figure 4** illustrates the inhibitory curves of hu8D6 (solid) and chimeric 8D6 (c8D6, dot line) to compete with HRP-conjugated c8D6 to bind human IgE by ELISA.

[0018] **Figure 5** illustrates the surface plasmon resonance (SPR) curves of hu8D6 Fab to human IgE by BIAcore analysis. RU: response unit.

[0019] **Figures 6a and 6b** illustrate the binding of hu8D6 and c8D6 to mIgE.Fc-expressing Ramos cell lines by flow cytometric analysis. mIgE.Fc_L Ramos: Ramos cells expressing the long form of human membrane-bound IgE.Fc (Fig 6a); mIgE.Fc_S Ramos: Ramos cells expressing the short form of human membrane-bound IgE.Fc (Fig 6b).

[0020] **Figure 7** illustrates the binding of hu8D6 to IgE-saturated recombinant α chain of human FcεRI.

[0021] **Figures 8a to 8e** illustrate the binding of hu8D6 to human FcεRI-expressing RBL SX-38 cells that were pre-loaded with human IgE.

[0022] **Figure 9** illustrates the inhibitory curves of hu8D6 and Omalizumab to compete with recombinant FcεRIa-Fc protein to bind human IgE by ELISA.

[0023] **Figure 10** illustrates the inhibitory curves of hu8D6 and Omalizumab to compete with native FcεRI receptor on RBL SX-38 cells for human IgE binding by flow cytometric analysis.

[0024] **Figure 11** illustrates the inability of hu8D6 to activate and trigger the degranulation of IgE-sensitized RBL SX-38 cells.

[0025] **Figure 12** illustrates the binding of hu8D6 to IgE-saturated recombinant trimeric human CD23.

[0026] **Figures 13a to 13d** illustrate the binding of hu8D6 to human CD23-expressing SKW6.4 cells.

[0027] **Figures 14a to 14e** illustrate the inability of the hu8D6:IgE immune complexes to bind to human FcεRI-expressing RBL SX-38 cells.

[0028] **Figure 15** illustrates the inability of the hu8D6:IgE immune complexes to activate and induce the degranulation of IgE-sensitized RBL SX-38 cells.

[0029] **Figures 16a to 16d** illustrate the binding of the hu8D6:IgE immune complexes to human CD23-expressing SKW6.4 cells.

[0030] **Figures 17a and 17b** illustrate the inhibitory effects of Omalizumab (Fig 17a) or hu8D6 (Fig 17b) on de novo IgE synthesis in vitro.

DETAILED DESCRIPTION OF THE INVENTION

[0031] The present invention pertains to humanized antibodies that bind to (1) human IgE in its secreted free form, (2) to membrane-bound IgE on B lymphocytes, and to (3) IgE bound by CD23 on B cells and (4) by free CD23 in solution. The anti-IgE antibodies do not bind to IgE bound by FcεRI on mast cells and basophils and hence do not activate those inflammatory cells.In a broad definition, a humanized antibody is one in which the constant domains of the heavy and light chains and the framework regions of the V_H and V_L domains are of human origin.

[0032] Various methodologies employing phage, yeast, or ribosomes to display single-chain antibody libraries, which comprise the V_H and V_L segments of human B lymphocytes, have been used successfully for screening antibodies for human cell surface antigens or cellular factors. Such methodologies can also be employed to screen human antibodies with the unique set of IgE-binding specificities of the present invention. Alternatively, antibodies of the present invention may be obtained in transgenic mice which bear human immunoglobulin VH and VL gene pools.

[0033] As an example, the present invention provides the detailed description of the preparation of a humanized version of murine anti-IgE monoclonal antibody, 8D6,

which possesses the above set of binding specificities. The present disclosure provides a humanized anti-human IgE antibody, hu8D6, comprising a heavy chain variable region (SEQ ID NO: 2) and a light chain variable region (SEQ ID NO: 4). The amino acid sequences of hypervariable regions of hu8D6 are identified as followed: CDR-H1 comprises GYTFNGYWMH (SEQ ID NO: 5); CDR-H2 comprises YINPTTGHTEYNQKFKD (SEQ ID NO: 6); CDR-H3 comprises ARQEYRHSWFAY (SEQ ID NO: 7); CDR-L1 comprises QSVDYDGDTY (SEQ ID NO: 8); CDR-L2 comprises AASNLDS (SEQ ID NO: 9); CDR-L3 comprises QQTNEDPWT (SEQ ID NO: 10).

[0034] The invention provides a humanized anti-human IgE antibody, hu8D6, wherein the binding activities of the antibody in its bivalent form to human IgE is EC_{50}=~10^{-10} M. The affinity of the antibody in its monovalent Fab form to human IgE is KD=~10^{-11} M.

[0035] In the following examples, the invention provides a humanized anti-IgE antibody, hu8D6, which is capable of binding to free human IgE (unbound by its two receptors), membrane-bound IgEs (long and short forms) on B lymphocytes, and CD23-bound IgE but incapable of binding to FcεRI-bound IgE and inducing - hexosaminidase release from mast cells or basophils. Besides, hu8D6 is able to efficiently inhibit the binding of IgE to FcεRI. The immune complex of hu8D6 and human IgE is capable of binding to CD23, but incapable of binding to FcεRI and inducing -hexosaminidase release from mast cells or basophils. Furthermore, hu8D6 can bind both membrane-bound IgE and crosslink CD23-bound IgE on B cells and hence can inhibit the de novo synthesis of IgE production from human peripheral mononuclear cells in an in vitro culture system.

[0036] In one aspect, the humanized anti-IgE antibody, hu8D6, can be a full-length antibody (e.g., an IgG molecule), an antigen-binding fragment (e.g., Fab or $F(ab')_2$), or a single-chain Fv.

[0037] In another aspect, the present humanized anti-IgE antibody, hu8D6, exhibits therapeutic properties and provides the method for treating IgE-mediated diseases, comprising administering to a patient in need thereof of an effective amount of the humanized anti-IgE antibody described herein either the antibody or the antigen-binding fragment. IgE-mediated diseases include allergic asthma, allergic rhinitis, atopic dermatitis, food allergy, chronic spontaneous (idiopathic) urticaria, chronic rhinosinusitis, systemic mastocytosis, cutaneous mastocytosis, allergic bronchopulmonary aspergillosis, recurrent idiopathic angioedema, and eosinophil-associated gastrointestinal disorder. It is noted that some of those diseases, such as chronic spontaneous urticaria, systemic mastocytosis, and recurrent idiopathic angioedema, are not allergic diseases and do not involve external allergens.

[0038] **EXAMPLE 1 GENERATION OF A HUMANIZED ANTI-IgE ANTIBODY**

[0039] **CDR grafting**

[0040] The amino acid sequence of V_H (SEQ ID NO: 1) and Vκ (SEQ ID NO: 3) domains from 8D6 were aligned with those of the human germline V_H1-69/JH4 and Vκ1-39/Jκ1 alleles. To make the CDR graft, the acceptor V_H framework, which differs from the human germline V_H1-69 allele at 4 positions M48I, R66K, V67A, and S76N, was used. The CDRs, which are position 26-35 (CDR-H1, SEQ ID NO: 5), 50-56 (CDR-H2, SEQ ID NO: 6), and 93-104 (CDR-H3, SEQ ID NO: 7) of 8D6 were engineered into the acceptor V_H framework to generate a direct CDR-graft of 8D6 V_H as shown in **Fig. 1**. In the Vκ domain,

the CDRs of positions 27-34 (CDR-L1, SEQ ID NO: 8), 50-56 (CDR-L2, SEQ ID NO: 9), and 89-97 (CDR-L3, SEQ ID NO: 10) were grafted to the acceptor Vκ framework, which differs from the human germline Vκ1-39 allele at a position M4L (**Fig. 2**). The direct CDR-graft of 8D6 V_L was referred to as hu8D6 V_L (SEQ ID NO: 4).

[0041] **IgG production**

[0042] In order to obtain humanized 8D6 (hu8D6) and chimeric 8D6 (c8D6) antibodies, the Expi293™ expression system (Invitrogen) was used for transient expression. Transfection was performed in 30 mL culture in 125 mL flasks using ExpiFectamine™ 293 reagent (Invitrogen). One day prior to transfection, Expi293F™ cells were diluted with Exppi293™ Expression medium (Invitrogen) to a density of 2 x 10^6 cells/mL. On the day of transfection, cultures were counted and concentrated to 2.5 x 10^6 cell/mL by centrifuging, removing old media, and adding fresh medium. Transfection complexes were made by diluting 80 µL of ExpiFectamine 293 into 1.5 mL of OPTI-MEM (Invitrogen) and 5 minutes alter adding the diluted ExpiFectamine 293™ solution to 30 µg plasmid DNA. 3 mL DNA-transfection reagent complex solution were then incubated at room temperature for 20 minutes and slowly added to the suspension culture while slowly swirling the flask. The transfected cells were placed back on the orbital shaker in a 37°C incubator and incubated for 16 hours. After incubation, 150 µL of ExpiFectamine 293™ Transfection Enhancer 1 and 1.5 mL of ExpiFectamine 293™ Transfection Enhancer 2 were added to the culture. The cultures were ended at day 7 post-transfection and harvested for antibody purification. Antibodies were purified from the supernatants using rProtein-A Sepharose™ affinity chromatography (GE Healthcare). The final product

was homogenous as determined by size exclusion fast protein liquid chromatography with $>$99% of the protein eluting as a single peak of about M_r 150,000.

[0043] **Binding analysis (EC50)**

[0044] The Fc region of human IgE (ε.Fc 2-4), which starts at Ser^{226} to Gly^{599} according to the numbering system as previously described by Wan et al. (*Nature Immunol.*, 2002, 3:681-686) and contains two mutations N265Q and N371Q, was expressed in Expi293F™ cells and purified by anti-IgE immunoaffinity chromatography using Omalizumab-coupled NHS-activated Sepharose™ resin (GE Healthcare). For EC_{50} determination of hu8D6, ε.Fc 2-4 protein was immobilized on 96-well plates at 50 ng/well in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated at 4°C overnight. Coated wells were blocked by 200 µL/well of assay diluents (0.5% BSA, 0.05% Tween-20, 0.01% thimerosal in PBS) at room temperature for 1 hour. Plates were washed 3 times with 200 µL/well of wash buffer (PBS with 0.05% Tween-20). 100 µL of antibody diluents (serially diluted from 0.1 mg/ml in 1:3 steps) were added to coated wells. The incubation was carried out at room temperature for 2 hours. All wells were aspirated and washed 6 times with 200 µL/well of wash buffer. The plates were incubated with a 1:10,000 dilution of horse radish peroxidase (HRP)-conjugated goat anti-murine IgG-Fc antibody (Jason ImmunoResearch) for 1 hour (100 µL/well). Then all wells were washed six times with 200 µL/well of wash buffer. Finally, wells were developed by 50 µL/well of NeA-Blue TMB substrate (Clinical Science Products) and the reaction was stopped by addition of 50 µL/well of 1N HCl. The absorbance was measured at OD_{450} on an ELISA reader. The EC_{50} was calculated using the Prism software (GraphPad). As shown in **Fig. 3**, the EC_{50} of

hu8D6 and c8D6 were 1.48 x 10^{-10} and 1.16 x 10^{-10} M. The results indicate that the binding activity of hu8D6 was almost completely preserved after CDR grafting process.

[0045] **Competition analysis (IC50)**

[0046] Competitive ELISA tests were performed to compare the effectiveness of hu8D6 and c8D6 to compete with HRP-conjugated c8D6 in binding to human IgE to determine the binding property of hu8D6. The wells of ELISA plates were coated with 50 ng of ε.Fc 2-4 protein in coating buffer overnight at 4°C and blocked by incubating with assay diluents. Separately, a stock solution of HRP-c8D6 was diluted 1:1,000 in assay diluents and aliquots were mixed with unconjugated c8D6 or hu8D6 serially diluted from 0.1 mg/ml in 1:3 steps. Both series of mixtures were added to the ELISA wells and incubated for 1 hour at room temperature. The wells were then washed with PBST, incubated with TMB, and their OD_{450}. The IC_{50} was calculated using the Prism software. The IC_{50} of unconjugated hu8D6 and c8D6 were 1.79 x 10^{-9} and 2.28 x 10^{-9} M as shown in **Fig. 4**. The results indicated that the hu8D6 bound to an identical epitope to 8D6.

[0047] **KD determination by surface plasmon resonance (SPR) analysis**

[0048] To produce hu8D6 Fab, cDNAs of V_H and V_k DNA segments of hu8D6 antibody were respectively joined to a human C 1-CH1 domain and a human C region of an Fab expression vector that was modified from the pIgG1( ) vector by replacing the entire C 1 constant region with the C 1-CH1 domain (Chen et al. *J Immunol.* 184:1748-1756). The Expi293™ expression system (Invitrogen) was used for transient expression. Hu8D6 Fabs was purified from the supernatants using KappaSelect Sepharose™ affinity chromatography (GE Healthcare). The final product was homogenous as determined by

size exclusion fast protein liquid chromatography with >99% of the protein eluting as a single peak of about M_r 50,000.

[0049] For KD determination of hu8D6, SPR assays were performed with a Biacore X instrument (GE Healthcare). Hu8D6 Fab was immobilized on a CM5 chip (GE Healthcare) by using an amine coupling kit (GE Healthcare). Coupling density was limited to <500 resonance units. ε.Fc 2-4 protein was injected over the sensor chip at concentrations of 25, 12.5, 6.25 and 3.125 nM in HBS-P buffer (GE Healthcare) at a flow rate of 30 µl min^{-1}. All samples were injected onto the flow cell for 120 s with a dissociation time of 720 s at 25 °C. Regeneration of the sensor surface was performed with two 30-second injection of 10 mM glycine-HCl (pH 2.5). The sonograms of ε.Fc 2-4, at different concentrations, binding to immobilized hu8D6 Fab were shown in **Fig. 5**. Affinity and rate constants were calculated by using BIAevalution software (GE Healthcare), showing that the k_{on} and k_{off} of hu8D6 Fab to ε.Fc 2-4 are 1.23±0.17x10^6 $M^{-1}s^{-1}$ and 5.79±0.03x10^{-5} s^{-1}, resulting in that the KD of hu8D6 is 4.8±0.7 x10^{-11} M.

[0050] **FACS analysis**

[0051] Ramos cell lines that were transfected with either recombinant DNA encoding mIgE.Fc_L (C_H2-cytoplasmic tail of the long form of membrane ε chain) or mIgE.Fc_S (C_H2-cytoplasmic tail of the short form of membrane ε chain) were generated by Chen et al. (Chen et al. *J Immunol.* 184:1748-1756) and used to determine binding activity of hu8D6. Ramos cells expressing mIgE.Fc_L or mIgE.Fc_S were resuspended in FACS buffer (1% FBS and 0.1% sodium azide in 1x PBS) at a cell density of 2 x 10^6 cells/mL. 2 x10^5 cells in 100 µL of FACS buffer were incubated with hu8D6 or c8D6 at the concentration of 10, 1, 0.1, 0.01 and 0.001µg/mL, for 30 minutes on ice, followed by washing with FACS buffer.

Bound antibodies were detected by FITC-labeled goat IgG specific for human IgG-Fc (Caltag Laboratories). The stained cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences). The geometric means of fluorescence intensity for the binding of antibodies to Ramos cell lines were analyzed using the FCSExpress software (DeNono software). The EC_{50} was calculated using the Prism software. As shown in **Figs. 6a and 6b**, The EC_{50} of hu8D6 and c8D6 to bind to mIgE.Fc_L-expressing Ramos cells were 2.21×10^{-10} and 2.0×10^{-10}, and the EC_{50} of hu8D6 and c8D6 to mIgE.Fc_S-expressing Ramos cells were 3.86×10^{-10} and 3.0×10^{-10}. The results indicated that the binding activities of hu8D6 and c8D6 to two mIgE isoforms were approximately equal.

[0052] **EXAMPLE 2 INABILITY OF hu8D6 TO BIND TO HUMAN FCεRI-BOUND IgE**

[0053] **Inability of hu8D6 to bind to IgE-saturated recombinant chain of human FcεRI**

[0054] ELISA was used to determine the binding of hu8D6 to solid-phase recombinant FcεRIα fusion protein (huFcεRIα-huFcγ1, referred to FcεRIα-Fc), which was composed of the extracellular region of human FcεRIα (from Val^{26} to Leu^{204}) and the hinge-C_H2-C_H3 portion of human γ1 (Shiung et al., *Immunobiology*, 2012, 217:676-683). The FcεRIα-Fc protein was expressed by the FreeStyl™ 393F system (Invitrogen) and purified by protein A chromatography. The wells of ELISA plates were coated with 2 μg/mL of FcεRIα-Fc in coating buffer at 4°C overnight and treated with assay diluents (0.5% BSA, 0.05% Tween-20, 0.01% thimerosal in PBS) for 1 hour at room temperature. The coated FcεRIα-Fc proteins were then saturated with 1 μg/mL IgE, which was purified from the culture medium of U266 cells by anti-IgE chromatography. After the wells were washed 6 times with wash buffer, the captured IgE was incubated with human IgE (Sigma),

hu8D6, c8D6, Omalizumab and a murine anti-human IgE mAb 5H2 (AbD Serotec) at 10, 1, 0.1, and 0.01 μg/mL. The bound human IgE and murine IgG were detected using HRP-conjugated goat anti-human kappa light chain (GeneTex) and HRP-conjugated goat anti-murine IgG.Fc (Jackson ImmunoResearch). Humanized 8D6, c8D6, and Omalizumab were not bound to IgE-saturated recombinant FcεRIα fusion protein, while 5H2 did do so at all concentrations as shown in **Fig. 7**.

[0055] **Inability of hu8D6 to bind to IgE on basophils**

RBL SX-38 cells, rat basophilic leukema cells transferred with genes encoding the α, β, and γ chains of human FcεRI (Wiegand et al., *J. Immunol.*, 1996, 157:221-230) were used as a pool of cell surface FcεRI. 2 x 10^6 RBL SX-38 cells in 1 mL of FACS buffer were incubated with IgE at the concentration of 3 μg/mL for 30 minutes on ice. The cells were washed with FACS buffer to remove unbound IgE and then 2 x 105 cells in 100 ml FACS buffer were incubated with hu8D6, c8D6, Omalizumab, and 5H2 antibody at the concentration of 10 μg/mL for 30 minutes on ice, followed by washing with FACS buffer. Bound antibodies were detected by FITC-labeled goat IgG specific for human IgG-Fc or FITC-labeled F(ab)'2 rabbit anti-murine IgG (AbD Serotec). The stained cells were analyzed on FACS Canto II. As shown in **Figs. 8a to 8e**, humanized 8D6, c8D6, and Omalizumab could not bind to IgE-saturated RBL SX-38 cells, and 5H2 was bound to RBL SX-38 cells.

[0056] **Competitive analysis to FcεRIα-Fc protein**

[0057] Competitive ELISA tests were performed to compare the effectiveness of hu8D6 and Omalizumab to compete with HRP-conjugated IgE in binding to FcεRIα-Fc protein (Shiung et al). The wells of ELISA plates were coated with 100 ng of U266 IgE

protein in coating buffer overnight at 4°C and blocked by incubating with assay diluents (0.5% BSA, 0.05% Tween-20, 0.01% thimerosal in PBS). Separately, a stock solution of HRP-IgE was diluted 1:6000 in assay diluents and aliquots were mixed with unconjugated hu8D6 or Omalizumab serially diluted from 0.1 mg/ml in 1:3 steps. Both series of mixtures were added to the ELISA wells and incubated for 1 hour at room temperature. The wells were then washed with PBST, incubated with TMB, and their OD_{450}. The IC_{50} was calculated using the Prism software. Trastuzumab (Herceptin™), which targets human HER2 protein as a negative control, did not inhibit the binding of IgE to FcεRIα-Fc. The IC_{50} of hu8D6 and Omalizumab to inhibit IgE binding to FcεRIα-Fc were 9.4 and 25.9 x 10^{-10} M as shown in **Fig. 9**, indicating that hu8D6 is superior to Omalizumab in inhibiting the binding of IgE to its receptor.

[0058] Competitive analysis to cell surface FcεRI

[0059] 2 x 10^5 RBL SX-38 cells in 0.1 mL of FACS buffer were incubated with hu8D6, Omalizumab or Trastuzumab at different concentrations of 45, 30, 13.3, 8.9, 5.93, 3.95, 2.63, 1.76, 1.17, 0.78, 0.52 and 0.35 µg/mL as well as FITC-labeled IgE (4.5 µg/mL) for 30 minutes on ice. The cells were washed with FACS buffer to remove unbound IgE. The stained cells were analyzed on FACS Canto II. The IC_{50} was calculated using the Prism software. Trastuzumab did not affect the binding of IgE to SX-38 cells. The IC_{50} of hu8D6 and Omalizumab to inhibit IgE binding to SX-38 cells were 18.8 x 10^{-9} and 24.7 x10^{-9} M as shown in **Fig. 10**, indicating that hu8D6 is superior to Omalizumab in inhibiting the binding of IgE to FcεRI-expressing cells.

[0060] **EXAMPLE 3 INABILITY OF hu8D6 TO SENSITIZE RBL SX-38 CELLS**

[0061] The extent of degranulation was assessed by measuring the release of β-hexosaminidase, a lysosomal enzyme stored in granules, into the culture medium. RBL SX-38 cells were seeded in a 24-well plate at 3 x 10^5 cells/well in 0.5 mL of culture medium overnight in at 37 °C. On the next day, the medium was removed and 0.25 mL of pre-warmed culture medium containing 1 μg/mL human IgE was added to each well. After 2 hour of incubation in a 37 °C incubator, each well was washed twice with 0.5 mL of Tyrode's buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, and 0.5 mg/mL BSA, pH 7.3) and then 0.25 mL of pre-warmed Tyrode's buffer containing 10, 1, 0.1, or 0.01 μg/mL of antibodies or 1% Triton X-100 added. After a 30 minutes incubation in a 37°C incubator, the supernatants were collected and centrifuged at 300xg for 5 minutes at room temperature and 50 μL clear supernatants were transferred from each well to that of a new 96 well black OptiPlate™ (Perkin-Elmer). 50 mL of substrate solution (80 μM of 4-MUG (4-methylumbelliferyl-N-acetyl-d-glucosaminide), Sigma) in 0.1 M citric acid buffer (pH 4.5) was added to each well and the plate was incubated at 37°C for 1 hour. The reaction was terminated by adding 100 μL glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7). The resulting fluorescence (excitation 355 nm; emission 460 nm) was measured at the top of each well by a Victor™ 3 fluorescence reader (Perkin-Elmer). Measured values were expressed as percentages relative to the value of release (100%) obtained by lysing cells with 1% Triton X-100. Humanized 8D6, c8D6, and Omalizumab did not induce the degranulation of IgE-sensitized RBL SX-38 cells, while 5H2 did so (**Fig. 11**).

[0062] **EXAMPLE 4 ABILITY OF hu8D6 TO BIND TO HUMAN CD23-BOUND IgE**

[0063] **Ability of hu8D6 to bind to IgE-saturated recombinant trimeric human CD23**

[0064] ELISA was used to determine the binding of hu8D6 to solid-phase recombinant human CD23 fusion protein (ILZ-CD23), which contains N-terminal isoleucine zipper (ILZ) and extracellular region (Asp^{48} to Ser^{321}) of human CD23 and forms a non-convalent trimer through the ILZ motif (Shiung et al., *Immunobiology*, 2012, 217:676-683). The polyhistidine-tagged ILZ-CD23 was expressed by the FreeStyle™ 293F system, purified from the transfection culture medium using nickel Sepharose™ chromatography (GE Healthcare), and then preserved in 1x HBSS buffer with 2 mM $CaCl_2$. ELISA plates were coated with purified ILZ-CD23 at 5 μg/mL and blocked, and the coated ILZ-CD23 was then saturated with 3 μg/mL of purified human IgE in Ca^{2+}/Mg^{2+} assay diluents (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.5% BSA, 0.05% Tween-20, and 0.01% thimerosal in PBS). After the wells were washed 6 times with wash buffer, captured IgE was then incubated with hu8D6, c8D6, Omalizumab and 5H2 at 10, 1, 0.1, and 0.01 μg/mL in Ca^{2+}/Mg^{2+} assay diluents. The bound human IgG and murine IgG were detected using HRP-conjugated goat anti-human IgG.Fc and HRP-conjugated goat anti-murine IgG.Fc. Humanized 8D6, c8D6 and 5H2 antibody, could bind to human IgE-saturated recombinant CD23 coated on solid surface, while Omalizumab has no binding activity, comparable to the isotype-control human IgG antibodies as shown in **Fig. 12**.

[0065] **Ability of hu8D6 to bind to IgE-pulsed SKW6.4 B cells**

[0066] A human B cell line expressing high level of CD23 (Pathan et al., Blood, 2008, 111:1594-1602) was used to pulse CD23 by incubating with IgE. 2 x 10^6 SKW6.4 cells in 1 mL of Ca^{2+}/Mg^{2+} FACS buffer (1mM $CaCl_2$, 0.5 mM $MgCl_2$, 1% FBS and 0.1%

sodium azide in 1X PBS) were incubated with IgE at the concentration of 3 μg/mL for 30 minutes on ice. The cells were washed with Ca^{2+}/Mg^{2+} FACS buffer incubated with hu8D6, c8D6, Omalizumab, and anti-CD23 antibody (Clone: EBVCS2, eBioscience) at the concentration of 10 μg/mL for 30 minutes on ice, followed by washing with Ca^{2+}/Mg^{2+} FACS buffer. Bound antibodies were detected by FITC-labeled goat IgG specific for human IgG-Fc or FITC-labeled $F(ab)'_2$ rabbit anti-murine IgG. The stained cells were analyzed on a FACSCanto™ II flow cytometer. Anti-CD23 was found to bind well onto the skw6.4 B cells as a positive control. Omalizumab was prevented from binding to IgE bound by CD23, while hu8D6 and c8D6 were able to effectively bind to IgE on SKW6.4 cells as shown in **Figs. 13a to 13d**.

[0067] **EXAMPLE 5 INABILITY OF THE IMMUNE COMPLEX OF hu8D6 AND IgE TO BIND TO HUMAN FcεRI**

[0068] Preparation of the immune complex of hu8D6 and IgE

[0069] Purified human IgE (about 190 kDa), hu8D6, c8D6, Omalizumab, and 5H2 (about 150 kDa) were diluted to 1 mg/mL in 1X PBS. Mixtures containing anti-IgE:IgE immune complexes were prepared by mixing 95 μL of 1 mg/mL human IgE, 75 μL of 1 mg/mL anti-IgE mAbs. The mixtures were incubated for 2 hours at room temperature and used for further use.

[0070] Inability of the immune complex of hu8D6 and IgE to bind to RBL SX-38 cells

[0071] 2 x 10^5 RBL SX-38 cells in 100 L FACS buffer were incubated with hu8D6:IgE, c8D6:IgE, Omalizumab:IgE, and 5H2:IgE immune complexes at the concentration of 10 μg/mL for 30 minutes on ice, followed by washing with FACS buffer.

Bound antibodies were detected by FITC-labeled goat IgG specific for human IgG-Fc or FITC-labeled $F(ab)'_2$ rabbit anti-murine IgG. The stained cells were analyzed on a FACSCanto™ II flow cytometer. As shown in **Figs. 14a to 14e**, the hu8D6:IgE, c8D6:IgE, and Omalizumab:IgE immune complexes were unable to bind to RBL SX-38 cells while as anti-FcεRI and 5H2:IgE did bind.

[0072] **Inability of the immune complex of hu8D6 and IgE to activate RBL SX-38 cells**

[0073] 3 x 10^5 RBL SX-38 cells were seeded in a 24-well plate in 0.5 mL of culture medium overnight in a 37°C incubator. On the next day, each well was washed twice with 0.5 mL of Tyrode's buffer and then 0.25 mL of pre-warmed Tyrode's buffer containing 10 µg/mL of the anti-IgE:IgE immune complexes or 1% Triton X-100 was added. After 30 minutes incubation at 37°C, the supernatants were collected and centrifuged at 300xg for 5 minutes at room temperature and 50 µL clear supernatant were transferred from each well to that of a new 96-well black OptiPlate™ (Perkin-Elmer). 50 µL of 4-MUG substrate solution was added into each well and the plate was incubated at 37°C for 1 hour. The reaction was terminated by adding 100 µL glycine buffer. As shown in **Fig. 15**, the hu8D6:IgE, c8D6:IgE, and Omalizumab:IgE immune complexes did not induce the degranulation of RBL SX-38 cells while as 5H2:IgE did.

[0074] **EXAMPLE 6 ABILITY OF THE IMMUNE COMPLEX OF hu8D6 AND IgE TO BIND TO HUMAN CD23**

[0075] 2 x 10^5 SKW6.4 cells in 100 µL of Ca^{2+}/Mg^{2+} FACS buffer were incubated with hu8D6:IgE, c8D6:IgE, Omalizumab:IgE immune complexes and anti-CD23 at the concentration of 10 µg/mL for 30 minutes on ice, followed by washing with Ca^{2+}/Mg^{2+}

FACS buffer. Bound anti-IgE:IgE immune complexes were detected by FITC-labeled goat IgG specific for human IgG-Fc or FITC-labeled $F(ab)'_2$ rabbit anti-murine IgG. The stained cells were analyzed on a FACS Canto™ II flow cytometer. As shown in **Figs. 16a to 16d**, the Omalizumab:IgE immune complex was not able to bind to surface CD23, whereas hu8D6:IgE, c8D6:IgE immune complexes and anti-CD23 were able to effectively bind to SKW6.4 cells.

[0076] **EXAMPLE 7 ABILITY OF hu8D6 TO INHIBIT IgE PRODUCTION OF PBMCs**

[0077] Peripheral blood mononuclear cells (PBMCs) were purified from blood samples from healthy donors or atopic patients by centrifugation over a Ficoll-Paque® PLUS (GE Healthcare) density gradient. The isolated PBMCs were suspended at 10^6 cell/mL in complete IMDM medium (Invitrogen) with 100 ng/mL human IL-4 (R&D systems) and 100 ng/mL mouse anti-CD40 monoclonal antibody (BioLegend, clone: G28-5). Humanized 8D6, Omalizumab and Trastuzumab were added to the medium at a concentration of 10 μg/ml. After 7-, 11- and 14-day cultivation, cell-free supernatants were harvested and stored at -20°C. IgE released into the supernatants were measured by ELISA using anti-human IgE, HP6061 (Abcam), as capture antibody and biotin-labeled anti-human IgE, HP6029 (Abcam), as detection antibody. Briefly, the HP6061 was immobilized on 96-well plates at 100 ng/well in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated at 4°C overnight. Coated wells were blocked by 200 μL/well of assay diluents (0.5% BSA, 0.05% Tween-20, 0.02% ProClin 300 in PBS) at room temperature for 1 hour. Plates were washed 3 times with 200 μL/well of wash buffer (PBS with 0.05% Tween-20). Purified U266 IgE was used to generate a standard curve (range 800 to 3.125 ng/ml by 2-fold serial dilution). To improve the accuracy of IgE qualification,

the IgE standards were prepared in complete IMDM medium and spiked with hu8D6, Omalizumab or Trastuzumab at 10 μg/ml. 100 μL of the clear supernatants and standards were added to coated wells. The incubation was carried out at room temperature for 1 hour. All wells were aspirated and washed 6 times with 200 μL/well of wash buffer. The captured IgE was incubated with 100 μL of detection antibody solution (50 ng/ml of biotin labeled HP6029 in assay diluent) at room temperature for 1 hour. Then, the bound biotin-HP6029 was detected using streptavidin poly-HRP (1: 10,000 dilution, Thermo Pierce) for 1 hour (100 μL/well). All wells were aspirated and washed 6 times with 200 μL/well of wash buffer. Finally, wells were developed by 100 μL/well of NeA-Blue TMB substrate (Clinical Science Products) and the reaction was stopped by addition of 100 μL/well of 1M H_2SO_4. The standard curve was created by using the SoftMax Pro software (Molecular Devices) to generate a four parameter logistic curve-fit and used to calculate the concentrations of IgE in all tested samples. Student t tests were used to compare data by using the Prism software. As shown in **Figs 17a and 17b**, hu8D6 at 10 mg/ml in the initiation of cultures reduced culture IgE levels by 88.1%, 93.4 %, and 88.6%, while Omalizumab reduced IgE levels by 21.3%, 26.4, and 30% at 7-, 11- and 14-day incubation, respectively. The results suggest that hu8D6 capable of binding membrane-bound and CD23-bound IgE on B cells is superior to Omalizumab, which doesn't bind IgE already bound by CD23, in inhibiting the de novo IgE synthesis.

CLAIMS

1. A humanized antibody, which binds to free IgE, membrane-bound IgE on B lymphocytes, or IgE bound by CD23, but not to IgE bound by FcεRI, wherein the humanized antibody comprises a heavy chain variable region (V_H) and a light chain variable region (V_L),

the V_H comprises an amino acid sequence that is identical to SEQ ID NO: 2, and the hypervariable regions of V_H are positions 26-35 (CDR-H1; SEQ ID NO: 5), 50-56 (CDR-H2; SEQ ID NO: 6) and 93-104 (CDR-H3; SEQ ID NO: 7); and

the V_L comprises an amino acid sequence that is identical to SEQ ID NO:4, and the hypervariable regions of V_L are positions 27-34 (CDR-L1; SEQ ID NO: 8), 50-56 (CDR-L2; SEQ ID NO: 9), and 89-97 (CDR-L3; SEQ ID NO: 10).

2. The humanized antibody of claim 1, which crosslinks IgE-bound CD23 on B lymphocytes.

3. The humanized antibody of claim 1, which decreases the total IgE production by B lymphocytes.

4. The humanized antibody of claim 1, which decreases the antigen-specific IgE production by antigen-activated B lymphocytes.

5. The humanized antibody of claim 1, which decreases IgE production in patients treated with the humanized antibody.

6. The humanized antibody of claim 1, wherein the antibody is an antigen-binding fragment.

7. The humanized antibody of claim 6, wherein the antigen-binding fragment is Fab, F(ab')2, or single-chain Fv.

8. A pharmaceutical composition, comprising a therapeutically-effective amount of the humanized antibody of claim 1 and a pharmaceutically acceptable carrier.

9. Use of a humanized antibody for the manufacture of a medicament for use in the treatment of a disease selected from the group consisting of allergic asthma, allergic rhinitis, atopic dermatitis, food allergy, chronic spontaneous (idiopathic) urticaria, chronic rhinosinusitis, systemic mastocytosis, cutaneous mastocytosis, allergic bronchopulmonary aspergillosis, recurrent idiopathic angioedema, and eosinophil-associated gastrointestinal disorder, wherein the humanized antibody is any of claims 1 to 7.

10. The use of claim 9, wherein at an effective amount of the humanized antibody, the humanized antibody does not induce the degranulation of mast cells and basophils.

11. The use of claim 9, wherein at an effective amount of the humanized antibody, the humanized antibody forms an immune complex with IgE, and the immune complex does not induce the degranulation of mast cells and basophils.