

Targeting the junction of C ϵ mX and ϵ -migis for the specific depletion of mIgE-expressing B cells

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ABSTRACT

Monoclonal antibodies targeting the extracellular region of the human IgE heavy chain membrane-tethering domain have been proposed for treating allergies caused by hyperproliferative monoclonal expansion of IgE-producing B cells. Antibodies against this target are expected to deplete membrane IgE (mIgE) displaying B cells and leave B cells of other immunoglobulin isotypes intact. Because of alternative splicing, the mIgE heavy chain has two isoforms that differ in their membrane-proximal segment. In the long isoform, the CH4 domain is followed by a 67-amino acid-long extracellular portion. Out of these 67 amino acids, the first 52 amino acids following the CH4 domain constitute the C ϵ mX segment while the rest of the 15 amino acids immediately adjacent to the membrane constitute the ϵ -migis. In the short isoform the C ϵ mX segment is absent and the CH4 domain is followed only by the 15-amino acid-long ϵ -migis segment. Using antibodies derived from a phage display library, we investigated: (1) ϵ -migis and (2) the junction of C ϵ mX and ϵ -migis (C ϵ mX.migis), as potential therapeutic antibody targets. Our results indicate that antibodies obtained from our phage library that target ϵ -migis bind to a variety of human cells irrespective of mIgE expression, possibly due to homology between ϵ -migis and a region of phosphoinositide-binding protein (ARAP3). In contrast, antibodies specific for the C ϵ mX.migis junctional region, bound specifically to transfected and primary B cells expressing human mIgE and elicited antibody-dependent cellular cytotoxicity and reduction in IgE production. These antibodies did not bind secreted IgE or the mIgE isoform in which C ϵ mX is absent. These results suggest that C ϵ mX.migis junctional region is a promising antibody target and the human antibodies we describe warrant further evaluation.

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1. Introduction

Diseases caused by hyperproliferative monoclonal expansion of B cells include allergies to specific antigens, several autoimmune diseases, and a variety of lymphomas. Clinical studies of the anti-B cell monoclonal antibody, rituximab for such conditions have been encouraging (Collins-Burow and Santos, 2007; Gurcan et al., 2009). However, rituximab depletes almost the entire B cell population, and the long-term immunologic effects of rituximab therapy are still not clearly understood (Anolik et al., 2007; Lu et al., 2008; St Clair, 2010). An ideal monoclonal antibody should eliminate only

the pathologic B cell subpopulation and leave normal B cell subpopulations unharmed.

Antibodies exist in two forms, namely, secreted form (sIg) and membrane bound form (mIg). mIg is a key component of B cell receptor (BCR) and is involved in antigen sensing, clonal expansion and differentiation (Parker, 1993). Membrane-bound immunoglobulin differs from secreted immunoglobulin by a peptide segment located at the C-terminus of the immunoglobulin heavy chain that tethers the immunoglobulin to the cell membrane. The membrane tethering region consists of three distinct regions. From inside out, the first is the intracellular region that differs for each immunoglobulin isotype and is involved in signal transduction by the B cell receptor (BCR). The second is a transmembrane (TM) region that is highly similar among immunoglobulin isotypes. The third tethering segment is the membrane-proximal extracellular segment that is unique for each immunoglobulin isotype and termed migis which is an acronym for Membrane ImmunoGlobulin Isotype Specific (Davis et al., 1991; Major et al., 1996). In fact, even before all

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the different migis sequences were determined, it was postulated that there is a unique epitope on mIgE which could be used to target mIgE displaying B cells (Chang et al., 1990). Because migis peptides are isotype specific, they are potential targets for treating B cell hyperproliferative disorders associated with specific B cell isotypes. Another attraction of this proposal is that membrane immunoglobulin expression, and hence migis peptide expression, spans B cell developmental stages from large pre-B cell to memory cell (Brown and Morrison, 1989).

Of the five human membrane immunoglobulin isotypes, mIgE is the most complex because of the existence of splice variants. Alternative splicing events produce two different isoforms of the mIgE heavy chain. In the short isoform, the CH4 domain of IgE is followed by the membrane-proximal 15-amino acid migis peptide, denoted ε -migis. In the long isoform, there is a 52-amino acid segment inserted between CH4 and ε -migis; this extra peptide is called the C ε mX region of the membrane tethering domain (Peng et al., 1992; Zhang et al., 1992). It has been known that mIgE plays a key role for IgE secretion in vivo by stimulating clonal expansion of specific B cells (Achatz et al., 1997). The role of sIgE is well established in the etiology of allergy, which has a large patient population and substantial unmet therapeutic needs. It was shown that a monoclonal antibody against the extracellular membrane-proximal domain of mouse membrane-bound IgE induces reduction in serum IgE in a mouse model of allergy by interfering with formation of antigen specific IgE memory B cells (Feichtner et al., 2008). This suggests that similar targeting strategy might be applied in human for treating allergies caused by hyperproliferation of IgE-producing B cells.

In our study, we applied a phage display approach and selected antibodies against ε -migis and the junction region of C ε mX and ε -migis (C ε mX.migis). In our phage approach targeting ε -migis, we failed to identify antibodies that could bind specifically to cells expressing mIgE. This is likely due to the high degree homology of ε -migis with a motif within another, possibly ubiquitous, membrane protein called ARAP3 (NCBI accession number BAF82741), which is a phosphoinositide-binding protein. On the contrary, we were able to identify phage antibodies that bind specifically to cells expressing mIgE by targeting C ε mX.migis. Two such antibodies were engineered to have an enhanced antibody-dependent cytotoxicity (ADCC) function and were able to deplete mIgE-displaying cells potently in vitro. One of these two antibodies, F4 was also evaluated in an ELISpot assay using primary human B cells and shown to reduce IgE positive spots. Our ADCC-enhanced antibodies targeting C ε mX.migis may have the potential to treat allergies and other pathologic conditions caused by hyperproliferation of IgE-producing B cells or formation of new IgE memory B cell (Kleinjan et al., 2000; Feichtner et al., 2008).

2. Materials and methods

2.1. Peptides

Peptides representing the extracellular membrane-proximal regions of mIgE were used to develop antibodies against the IgE ε -migis and C ε mX.migis junction. A hexaminoic acid linker (Ahx) and a biotinylated lysine residue (K-Biot) were attached to the C-terminal end of each peptide to ensure that the peptide would not be embedded in the biotin-binding pocket of streptavidin (Hendrickson et al., 1989) and hence became inaccessible to phage particles during panning. The following peptides were used:

- (1) ε -migis: NH₂-ELDVCVEEAEGEAPW-(Ahx)-(K-Biot)-COOH.
- (2) Human C ε mX.migis junction: NH₂-RADWPGPP ELDVCVEEAEGEAPW-(Ahx) (K-Biot)-COOH. This peptide consists of the last 8 amino acids of C ε mX followed by the 15

amino acids of ε -migis (in italics). Throughout the text this peptide will be referred as C ε mX.migis.

- (3) Cynomolgous C ε mX.migis junction: NH₂-RADWPGPP ELDLCVEEAEEVLPW-(Ahx) (K-Biot)-COOH. This peptide consists of the last 8 amino acids of cynomolgous C ε mX followed by the 15 amino acids of cynomolgous ε -migis (in italics). Throughout the text this peptide will be referred as cyC ε mX.migis.
- (4) Scrambled form of ε -migis peptide (control reagent): NH₂-GEDWCEVALEPAEVE-(Ahx) (K-Biot)-COOH.
- (5) Control peptide encompassing γ -migis: NH₂-KSLSLSP ELQLEESCAEAQDGLDG-(Ahx) (K-Biot)-COOH.
- (6) Control peptide encompassing μ -migis: NH₂-ERTVDKST EGEVSADEEGFEN-(Ahx) (K-Biot)-COOH.
- (7) ARAP3 peptide: NH₂-TQLLCVEAFEGEEPW-(Ahx) (K-Biot)-COOH. This peptide has high homology to ε -migis and was used to test cross-reactivity of anti- ε -migis antibodies.

All ε .migis and C ε mX.migis mentioned in the text are human sequences unless otherwise noted. All peptides except (4), (5), and (7) were dissolved in phosphate-buffered saline (PBS, pH 7.4). Peptides 4 and 5 were dissolved in 10% dimethyl sulfoxide (DMSO), and peptide 7 was dissolved in 5% DMSO. Of note, peptides 3, 4, and 6 were not soluble in PBS. The concentration of the stock solution of all peptides was 1 mg/ml.

2.2. Recombinant IgE protein expression and their use in binding study

Three different recombinant IgE proteins were made to test specificity of the antibodies that bound to ε -migis or C ε mX.migis peptides. They were: (1) soluble (or secreted) IgE (sIgE); (2) IgE with 52 amino acids corresponding to the C ε mX region at the C-terminus of the CH4 domain (IgE.C ε mX); and (3) IgE with 67 amino acids corresponding to the C ε mX and ε -migis regions at the end of CH4 domain (IgE.C ε mX.migis). The three proteins have the same variable domains that bind specifically to EphA2 (internal unpublished data). Among the three IgE proteins, besides the obvious differences due to C ε mX and C ε mX.migis, there are minor differences at the end of the CH4 domain. These CH4 differences correspond to the difference seen between sIgE and mIgE. In sIgE, CH4 ends with the sequence SVNPGK, and in IgE.C ε mX and IgE.C ε mX.migis, the CH4 domain ends with SVNPP. The heavy and light chains of each IgE protein were cloned into a mammalian expression vector under the control of separate CMV promoters. Unfortunately, the expression levels of the three IgE proteins were exceedingly low. Their presence in culture supernatants was monitored by a sandwich enzyme-linked immunosorbent assay (ELISA) in which they were captured with an anti-human IgE Fc-specific antibody (5 μ g/ml in PBS, pH 7.4) and detected with anti-human kappa antibody conjugated with horse radish peroxidase (HRP). Due to low expression, the IgE proteins were not purified and the culture supernatants containing them were used directly in binding assays. To study the specificity of the ε -migis and C ε mX.migis antibodies, the antibodies were immobilized onto ELISA plates at 5 μ g/ml in PBS (pH 7.4) and used to capture each of the three recombinant IgE proteins separately. The captured proteins were then detected with anti-human IgE Fc-specific antibody conjugated with HRP. An IgG1 antibody specific for the F-protein of respiratory syncytial virus, was used as a negative control in all studies.

2.3. Generation of mIgE-expressing cell line

Nucleoporation was used for generating transfected cell lines expressing mIgE on the surface. Cells (293T and NS0) were cotransfected with: (1) a linearized bicistronic mammalian expression

vector coding for the mIgE heavy chain and a κ -light chain; the variable domains of mIgE were derived from an antibody that binds the F-protein of respiratory syncytial virus; and (2) a linearized bicistronic mammalian expression vector coding for CD79a and CD79b. CD79a and CD79b associate with membrane immunoglobulins to form the BCR complex (Reth, 1994). Twenty-four hours after cotransfection, cells were seeded at 500 cells/well in 96-well plates and subjected to double selection with 500 μ g/ml neomycin (for the IgE-expressing plasmid) and 100 μ g/ml hygromycin (for the CD79a- and CD79b-expressing plasmid) in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen). After 2–3 weeks, colonies surviving double selection were expanded and tested for expression of mIgE, CD79a, and CD79b. Colonies with expression of all three were sorted by three-color fluorescence-activated cell sorting (FACS) into a single cell/well. Those clones with constant expression of mIgE, CD79a, and CD79b were then subcloned by limited dilution cloning at 0.2 cell/well to ensure monoclonality.

2.4. Panning the antibody phage library

An antibody phage library displaying human Fab fragments and shown to be an effective source of antibodies against a wide variety of antigens (Hoet et al., 2005) was used to pan against the ε -migus and C μ mX.migus peptides. In short, the library has an estimated size of about 3.5×10^{10} . The light chains of the library were derived from 35 autoimmune patients and the heavy chain is semi-synthetic in nature. FR1–FR3 are derived from VH3–23 germ line gene with mutations introduced in CDR1 and 2 to mimic the position and pattern of mutations seen in natural antibody sequences. The CDR3–FR4 was derived from the μ heavy chain of PBMCs from 35 autoimmune and 10 healthy donors. Both solid and solution phase panning were employed.

For solid phase panning, NeutrAvidin (Pierce) at 20 μ g/ml concentration in PBS (pH 7.4) was used to coat immunotubes, and these immunotubes were used to capture peptides. In the first two rounds of panning, 25 μ g/ml (13 μ M) solutions of the peptides in PBS (pH 7.4) were used, whereas 2.5 μ g/ml (1.3 μ M) was used in the third round. At each round the phage library was blocked with 3% bovine serum albumin (BSA) in TPBS (0.1% Tween 20 in PBS, pH 7.2) for 1 h, then deselected for NeutrAvidin binders by exposing the library to a tube coated with 2 mg/ml of NeutrAvidin for 2 h, followed by exposure to a tube coated with NeutrAvidin-biotinylated peptide complexes for 1.5 h. Panning tubes were then washed 15 times each with TPBS and PBS before eluting the bound phage with an aqueous solution of 100 mM triethylamine (Sigma) for 15 min. Eluted phage solutions were immediately treated with 0.5 M Tris (pH 8) and then titered and amplified by infecting *Escherichia coli* strain TG1 for the next round of panning.

For solution panning, 2 μ g (1 μ M) of the biotinylated peptides were used for the first two rounds, and 0.2 μ g (0.1 μ M) was used for the third round of panning. The phage library was first blocked and deselected on streptavidin-coated magnetic beads for 2 h before incubating it with the peptide for 1.5 h. The free peptide and phage-peptide complexes were then captured using streptavidin-coated magnetic beads. The beads were washed 7 times each with TPBS and PBS, each wash being of 2 min duration. Following washing, the phage was eluted using an aqueous solution of 100 mM triethylamine and treated as described above for immobilized panning.

2.5. Phage library screening

To screen for specificity, recombinant antibody phage particles from single bacterial colonies were rescued in 96-well formats as described before (Chowdhury et al., 1997). Bacterial cultures were cooled to 4°C, and the cells were removed by centrifugation at $5000 \times g$ for 15 min at 4°C. Centrifuged supernatants

containing recombinant phage particles were used for screening assays. To screen for peptide specificity, solutions of biotinylated peptides at 2.5 μ g/ml (1.3 μ M) in PBS (pH 7.4) were immobilized onto NeutrAvidin-coated (5 μ g/ml in PBS, pH 7.4) individual wells of 96-well plates. After blocking the wells with 3% BSA in TPBS, supernatants containing recombinant phage particles were added. After incubation for 60 min at ambient temperature, the wells were washed, and the bound phages were detected with anti-M13 antibody conjugated to HRP. Plates were developed with 3,3',5,5'-tetramethylbenzidine substrate solution (Pierce). The reaction was stopped with 2 N sulfuric acid and signal was measured at 450 nm.

2.6. Reformatting of phage Fab to whole IgG1

The Fab gene in the phage display vector consisted of the human antibody light chain coding sequence separated from the Fd fragment by a DNA segment with a bacterial ribosome binding site. For reformatting the Fab fragment selected from the phage library into human IgG1, the Fab cassette was isolated by digesting the phagemid with unique restriction enzymes (*Apa*LI and *Nhe*I) and ligated into a mammalian expression vector such that the Fd fragment ligated in-frame with the constant domains of the human IgG1 heavy chain encoded by the mammalian expression vector. The vector encodes for an IgG1 Fc which has three mutations (IgG1–3M) designed to increase the ADCC activity of the IgG (Bruckheimer et al., 2009). From the resulting plasmid, the bacterial ribosome binding site between the light and Fd encoding DNA was then removed by restriction enzyme digestion and replaced by an internal ribosome entry sequence (IRES) for mammalian expression systems. The final vector thus created is monocistronic; the light and heavy chains are transcribed into one mRNA but are separately translated into proteins.

2.7. Expression and screening of antibodies for binding to peptides and mIgE-expressing cells

Following conversion of Fab genes from phage clones into IgG1s in mammalian expression vectors, the IgG1s were expressed in 293T cells by transient transfection. Cells were transfected with the IgG1 expression vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions, and they were cultured for 72 h in DMEM with 10% ultralow IgG-containing FBS (Invitrogen). Antibody concentration in the conditioned media was estimated by a sandwich ELISA in which anti-human kappa antibody (Sigma) was used to capture human IgG from the conditioned culture media and anti-human IgG Fc antibody conjugated to HRP was used to detect the captured IgG. The amount of IgG present in the conditioned media was estimated by using purified human IgG1/ κ as a standard. Antibodies in conditioned supernatants were normalized to same concentration and were screened for binding to peptide by ELISA and to mIgE-expressing cells by FACS.

For ELISA screening for peptide specificity, solutions of biotinylated peptides at 2.5 μ g/ml (1.3 μ M) in PBS (pH 7.4) were immobilized onto NeutrAvidin-coated microtiter wells of 96-well ELISA plates. After blocking the wells with 3% BSA in TPBS, supernatants containing recombinant human IgG1 were added to blocked wells of the ELISA plates. After incubation for 60 min at ambient temperature, the wells were washed and IgG1 binding was detected with goat anti-human IgG Fc-specific antibody conjugated to HRP (Jackson Laboratories). ELISA plates were developed with 3,3',5,5'-tetramethylbenzidine substrate solution. The reaction was stopped with 2 N sulphuric acid and signal was measured at 450 nm.

For FACS screening, all steps were carried out at 4 °C. Each sample used 2×10^4 cells. Cells were either: (1) 293T cells; (2) 293T stably transfected with mIgE, CD79a, and CD79b; (3) human B cell lines IM-9, Raji, RPMI 1740, or Daikiki; or (4) T-cell line CCRF-CEM. The cells were first blocked with 2% BSA in PBS (blocker). They were then exposed to the conditioned media containing 2–5 μg IgG1 in 200 μl blocker for 45 min. Cells were washed three times using 5 ml blocker and centrifuged for 5 min at $400 \times g$. The cells were then stained with anti-human IgG1 Fc-specific antibody-labeled with Alexa Fluor 488 and analyzed in a Guava FACS machine. IgG1s that showed specific binding to mIgE-expressing cells were studied further by FACS staining in the absence and presence of the ϵ -migus peptide or a scrambled peptide at 20 $\mu\text{g}/\text{ml}$ in PBS (pH 7.4).

2.8. Competition ELISA for identifying phage clones that would bind specifically to C ϵ mX.migus peptide and to mIgE-expressing cells

One of the challenges faced in the study was the huge enrichment of undesirable clones from the phage library. Although these clones were specific to different epitopes on the target peptides they failed to show specific binding to target cells. To evaluate if there were any rare clones that met the desirable characteristic of binding specifically to mIgE expressing cells we employed competition screening of the phage output pool using the undesirable IgG clones. The rationale was to identify rare clones binding to different epitope(s) on the target. As competitors, A1c and B1 IgGs specific to two different undesirable epitopes were used. For this experiment, a solution of 2.5 $\mu\text{g}/\text{ml}$ of C ϵ mX.migus peptide in PBS (pH 7.4) was immobilized onto a Neutravidin-coated plate as described earlier. Recombinant phage particles (100 μl , titer of about 10^{10} cfu/ml) derived from panning against C ϵ mX.migus peptide were then added to ELISA wells along with an IgG specific to the F-protein of respiratory syncytial virus as a negative control, or with A1c or B1 IgG1 at 20 $\mu\text{g}/\text{ml}$ concentration. At the end of 60 min, the ELISA wells were washed, and anti-M13 IgG conjugated to HRP was added to detect bound phage. The plates were developed as described under the phage-screening assay.

2.9. Antibody-dependent cellular cytotoxicity assay

The antibody-dependent cellular cytotoxicity (ADCC) activity of the antibodies was assessed as follows: IRB approved human blood samples were collected from individual healthy donors using heparinized syringes. The blood samples were diluted with twice the volume of PBS buffer, layered onto a Lymphoprep gradient (ICN Biomedicals, Thame, UK), and centrifuged at $400 \times g$ for 30 min at room temperature. Peripheral blood mononuclear cells (PBMCs) were harvested from the interface, washed three times with PBS (pH 7.4), and resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (Invitrogen) supplemented with 10% FBS. After recombinant human IL-2 (40 ng/ml) (R&D Systems,) was added, the PBMCs were incubated overnight at 37 °C in T-175 flasks (BD Biosciences, Bedford, MA). Untransfected 293T cells and 293T cells expressing mIgE, CD79a, and CD79b were harvested using cell dissociation buffer (Invitrogen) and resuspended in ADCC assay buffer (RPMI 1640 supplemented with 5% FBS) at a density of 2×10^5 cells/ml. The cells were then added to a 96-well round bottom tissue culture plate (BD Biosciences) at 50 $\mu\text{l}/\text{well}$, along with various concentrations of antibody at 50 $\mu\text{l}/\text{well}$ in ADCC assay buffer and preincubated at 37 °C for 30 min. PBMCs were then harvested from overnight incubation and resuspended at 2.5×10^6 cells/ml (for an Effector (E):Target (T) ratio of 25:1) in ADCC assay buffer and added at 100 $\mu\text{l}/\text{well}$ to the assay plate and incubated for 4 h. ADCC activity of anti-C ϵ mX.migus antibodies with mIgE-expressing NS0 cells as targets was tested at different doses

of the antibodies. A transformed NK cell line was used as effector cell at an E:T ratio of 2.5:1. Cells were incubated with serial dilutions of antibody for 5 h. Target cell lysis was measured by detecting the release of LDH. All assays were done in triplicate. For maximum cell lysis, 25 $\mu\text{l}/\text{well}$ of 9% Triton X-100 (Promega) was added to the control wells. The plates were centrifuged at $300 \times g$ for 3 min and incubated at 37 °C for 4 h. Plates were then centrifuged at $300 \times g$ for 10 min and 50 μl of supernatant from each well containing lactate dehydrogenase (LDH) released from lysed cells was transferred to MaxiSorp 96-well plates (BD Biosciences). Next, 50 μl of reconstituted substrate mix (CytoTox 96 Non-Radioactive Cytotoxicity Assay kit, Promega) was added to wells, and all was incubated in the dark at room temperature for 30 min. Reactions were terminated with 50 μl stop solution (Promega). LDH activity was quantified by measuring the absorbance at 490 nm. Percent cytotoxicity was calculated as follows:

Percent cytotoxicity =

$$\frac{(\text{experimental} - \text{effector spontaneous} - \text{target spontaneous})}{(\text{target maximum} - \text{target spontaneous})} \times 100,$$

where: “experimental” corresponds to the signal measured in experimental wells; “effector spontaneous” corresponds to the signal measured in the presence of PBMCs alone; “target spontaneous” corresponds to the signal measured in the presence of 293T (transfected or untransfected) target cells alone; “target maximum” corresponds to the signal measured in the presence of detergent-lysed 293T cells (transfected or untransfected).

2.10. ELISpot assay for antibody dependent reduction of IgE B cells obtained by class switching of PBMCs from healthy donors

IRB approved human blood samples were collected from individual healthy donors using sodium citrate cell preparation tubes. These were centrifuged at $1700 \times g$ for 25 min at room temperature. The red blood cells were predominantly beneath the density gradient, and the supernatant, containing peripheral blood mononuclear cells (PBMC), was decanted. PBMCs were centrifuged at $524 \times g$ for 7 min and the supernatant was aspirated. Pellets were resuspended in 10 ml of complete media (RPMI minus phenol red, 2% Penicillin Streptomycin, 1% L-glutamine, 0.1% β -mercapto ethanol, 10% fetal bovine serum, and 0.5% HEPES). Cells were then centrifuged for 10 min at $200 \times g$ with the brake off. Supernatants were aspirated and residual red blood cells were lysed in ACK

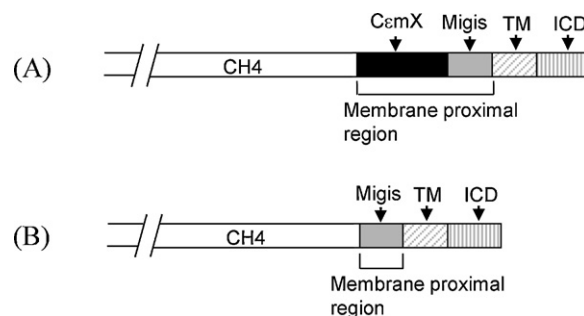


Fig. 1. Schematic representation of different membrane tethering domains in two mIgE heavy chain isoforms. (a) In the long form, the mIgE is tethered to the cell membrane by a 67 residue membrane proximal segment that consists of a 52 residue long region called C ϵ mX and a 15 residue long segment called ϵ -migus. The ϵ -migus is followed by the transmembrane (TM) and intracellular segments (ICD) of mIgE. (b) In the short form, C ϵ mX region is missing and the mIgE is tethered to the cell membrane by only the ϵ -migus region followed by the transmembrane and the intracellular segments. The two forms result from alternative splicing of mIgE expressing mRNA.

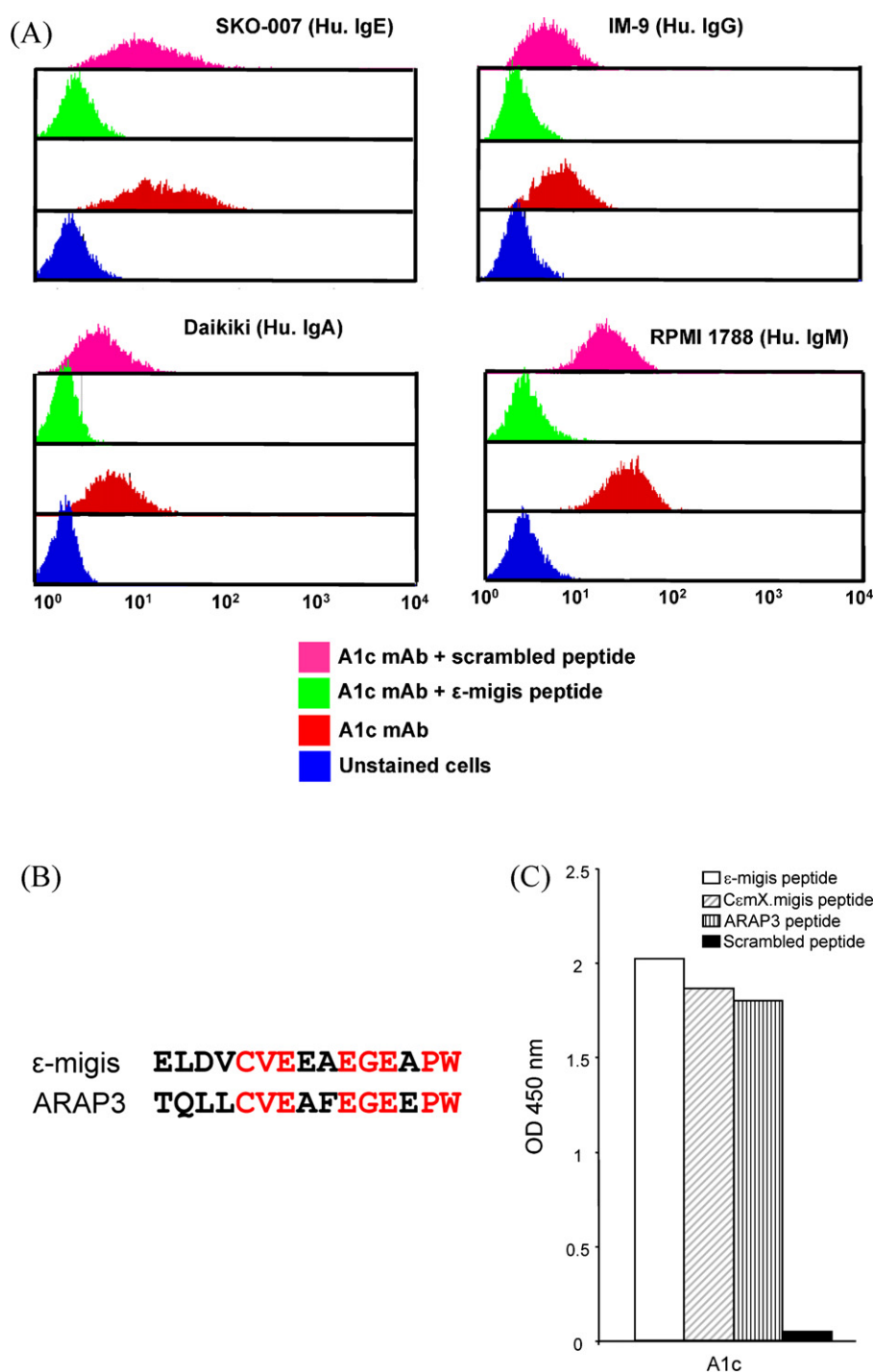


Fig. 2. (A) FACS experiment showing that anti- ϵ -migis IgG, A1c, bound to human cells irrespective of the presence or absence of mIgE. Cell lines expressing mIgE (SKO-007), IgG (IM-9), IgA (Daikiki) and IgM (RPMI 1788) were treated with 10 μ g/ml of A1c followed by Alexa Fluor 488-labeled anti-human IgG Fc (red). In green and pink are samples containing either the ϵ -migis or a scrambled peptide along with the A1c antibody. (B) Amino acid sequence alignment between ϵ -migis and ARAP3 peptide showing high similarity. (C) ELISA results showing that A1c bound to migis, CεmX.migis, and ARAP3 peptide, but not to scrambled peptide.

lysis buffer (Life Technologies) according to the manufacturer's instructions. Lysis was quenched in less than 5 min with the addition of 10 ml of complete media. Cells were centrifuged for 5 min at $524 \times g$. Cell pellets were resuspended in 10 ml of complete media and passed through a 50μ m filter. Freshly isolated PBMCs (1×10^6 cells/well) were IgE class switched by incubation with IL4 (10 ng/ml) and anti-CD40 (1 μ g/ml) and co-incubated with 10 μ g/ml of either isotype control or anti-migis F4IgG1-3M at 37°C for 7 days in a 5% CO_2 incubator. Following 7 day incubation,

cells were washed two times in complete media and transferred to Mabtech IgE ELISpot plates and incubated for 24 h at 37°C in a 5% CO_2 incubator. The Mabtech IgE ELISpot protocol was followed for IgE ELISpot development. The numbers of IgE positive spots were compared between the negative control antibody and the test antibody. ELISpot images and spot quantitation was performed using a BioReader 5000-Fx from Biosys. The experiment was done with 6 different donors and 6 replicates for each condition.

3. Results

3.1. Isolation of anti- ϵ -migris antibodies

The study commenced with the hypothesis that ϵ -migris might be a potential target for developing therapeutic human antibodies to specifically target mlgE-expressing B cells without affecting other B cell isotypes. ϵ -migris is a 15-amino acid-long membrane-proximal segment intercalated between IgE CH4 and the TM domain (Fig. 1). Because human mlgE is an integral membrane protein that is difficult to purify, a human ϵ -migris peptide was used to pan and screen a human Fab phage display library. After three rounds of panning we got 18 unique binders which were subsequently converted into full length human IgG1. When characterized by FACS, only 1 of the 18 clones, named A1c, was able to bind to mlgE-expressing cells. The binding of A1c to mlgE-expressing cells could be inhibited specifically by the ϵ -migris peptide but not by a control scrambled peptide (Fig. 2A). Upon further analysis we found that A1c bound to many different human cells irrespective of the presence or absence of mlgE and this binding could be inhibited by the ϵ -migris peptide but not by the scrambled peptide (Fig. 2A). This suggested that the epitope for the A1c antibody was probably present on another membrane antigen ubiquitous among the human cells tested. Indeed, a database search revealed that a region of the membrane protein ARAP3, which plays a role in anthrax pathogenesis (Abrami et al., 2003; Lu et al., 2004), had high sequence homology with the 15-amino acid-long ϵ -migris peptide (Fig. 2B). An ELISA assay revealed that A1c bound ϵ -migris peptide and the homologous ARAP3 peptide with nearly equal efficiency (Fig. 2C). In contrast, the other 17 antibody clones that bound to ϵ -migris peptide but not to mlgE-expressing human cells did not bind the ARAP3 peptide (data not shown). Since A1c was the only clone from our library that bound to human cells and failed to show specificity for mlgE-expressing cells, we decided to explore different areas in the membrane-proximal region of mlgE. It is, however, possible that there are other epitopes on ϵ -migris and antibodies against them may be able to bind mlgE-expressing cells specifically and that we were unable to find those antibodies from our library after significant efforts.

3.2. Isolation of antibodies against the junction between C ϵ mX and ϵ -migris

mlgE is known to have two different isoforms as a result of alternative mRNA splicing events. Existing literature indicates that in mlgE-expressing B cells and myelomas the long isoform (Fig. 1A) consisting of the C ϵ mX and migris is the predominant form of the protein (Peng et al., 1992). Using our phage library we had tried to isolate antibodies against recombinant IgE.C ϵ mX and had found that the only antibodies that we could isolate were at best weak binders and directed against the C-terminus of C ϵ mX (data not shown). This suggested that most of the C ϵ mX was probably inaccessible to the phage library at the conditions we used. We then decided to explore the junction region of C ϵ mX and ϵ -migris. A C ϵ mX.migris junction peptide containing the last 8 amino acids of C ϵ mX and the 15 amino acids of ϵ -migris was synthesized and used for solid and solution phase panning of the phage library. After three rounds of panning, antibody phage clones from 2500 individual bacterial colonies were screened by peptide ELISA, of which 108 were found to bind to the C ϵ mX.migris peptide, but not to ϵ -migris or other control peptides (peptides 4–6 in Section 2) (data not shown). From this group, 20 phage clones were randomly picked and reformatted to full IgG1. The binding pattern of the 20 IgG1s fell into two categories. 15 IgG1s bound to all human B cell lines similar to the anti- ϵ -migris antibody A1c. The other

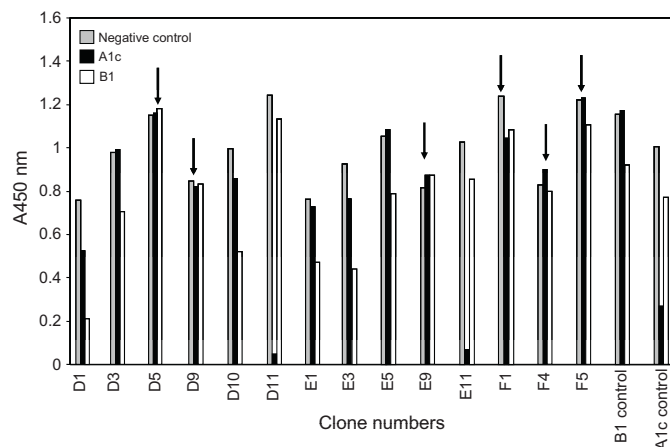


Fig. 3. Competition ELISA screening of phage clones with A1c and B1 IgG for binding to C ϵ mX.migris peptide. Individual phage clones were added to C ϵ mX.migris-immobilized wells either in the presence of a control antibody (gray), A1c (black), or B1 (white). The plates were developed with anti-phage IgG–HRP conjugate. Clones marked by arrows are examples of those that are not inhibited by A1c and B1.

5 antibodies did not bind any of the cells tested. Clone B1 is typical of this class.

3.3. Screening for antibodies that bind mlgE-expressing 293T cell line

Results of FACS screening assays employing the first 20 IgGs that were reformatted from phage clones specific for C ϵ mX.migris peptide showed two undesired binding patterns (described above). These two types of clones were probably dominant among the selected phage population. Therefore to screen for the rare phage clones that would bind to epitopes distinct from these two groups, we developed an ELISA assay wherein we used A1c and B1 IgG1s as competitors for phage binding to C ϵ mX.migris peptide. As shown in Fig. 3, D11 and E11 are examples of phage clones inhibited by A1c IgG1, whereas samples D1, E1 and E3 are examples of phage clones inhibited by B1 IgG1. Clones such as D5, D9, E9, F1, F4, and F5 are examples of ones that are not inhibited by either A1c or B1. As a result of this re-screening, 25 out of the 108 C ϵ mX.migris-positive phage clones were not inhibited by A1c or B1. Fab genes from these phage clones were then reformatted to full human IgG1s for further characterization.

Cell line SKO-007 is reported to express mlgE; however, we found that mlgE expression on SKO-007 cell was not only very weak but also inconsistent (data not shown). Therefore for proper screening of the antibodies, we had generated a 293T cell line that reliably expresses mlgE. Because efficient and functional expression of membrane immunoglobulins requires the presence of CD79a (Ig- α) and CD79b (Ig- β) (Reth, 1994), we generated a 293T cell line that co-expressed mlgE, CD79a and CD79b in a stable manner (described in Section 2). The expression of all three components on stable cells was verified by FACS (Fig. 4A). The variable domain for the human mlgE in these cell lines was derived from an antibody that binds to the F-protein of respiratory syncytial virus and had no reactivity with human cells or tissues. The 25 converted IgGs were tested for binding to this new cell line. Two of the antibody clones, D5 and F4, were found to bind selectively to the mlgE-expressing 293T cells with no or little binding to untransfected 293T cells (Fig. 4A). The two antibodies also did not show binding to other human cell lines like Daikiki, RPMI 1788 and CCRF-CEM (Fig. 4B). Of note, the binding that was seen in Daikiki cells was due to the non-specific binding of the secondary antibody. In Fig. 5 we showed that in contrast to

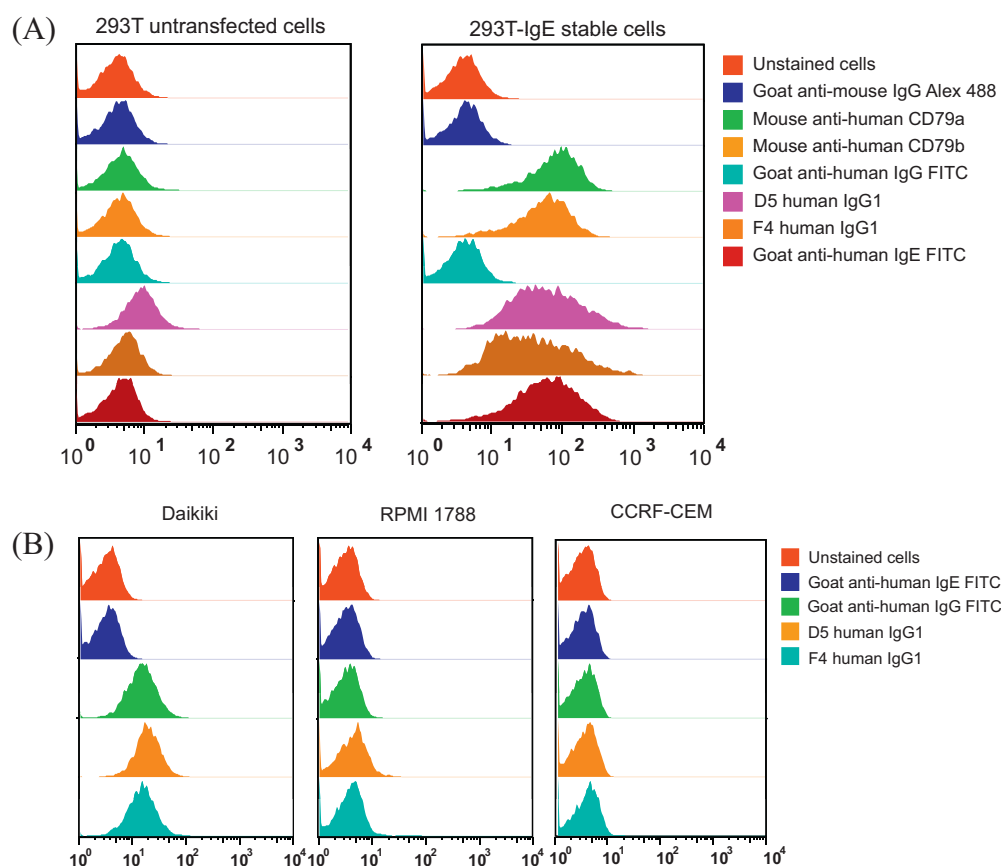


Fig. 4. (A) FACS assay showed that D5 and F4 IgG bound specifically to a 293T cell line that were stably transfected with human IgE, CD79a and CD79b but not to untransfected 293T cells. On the transfected 293T cell line, expression of human IgE was shown by staining with a goat polyclonal anti-human IgE antibody conjugated to FITC; expression of CD79a and CD79b was shown by staining with mouse anti-human CD79a and CD79b monoclonal antibodies. (B) D5 and F4 IgG did not bind to human cell lines Daikiki, RPMI 1788 and CCRF-CEM. The apparent binding to Daikiki is due to the non-specific binding of the goat anti-human IgG FITC secondary antibody.

A1c, D5 and F4 phage clones bound to C ϵ mX.migis peptide, but not to ϵ -migis or the related peptide from ARAP3.

3.4. Antibody D5 and F4 binding to mIgE requires regions of the membrane tethering domain

To further characterize the binding property of D5 and F4 IgGs, we tested their binding to three soluble forms of recombinant IgE by ELISA. The three forms are: (1) soluble IgE (sIgE); (2)

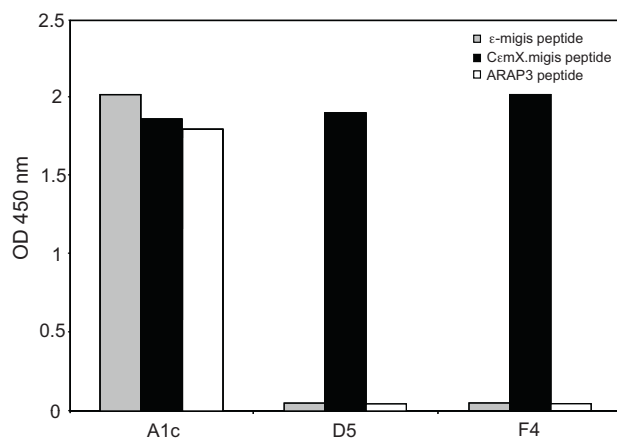


Fig. 5. ELISA data showing D5 and F4 Fab-displaying phages, bound specifically to C ϵ mX.migis but not to ϵ -migis and ARAP3 peptides. In contrast, A1c phage clone bound to all three peptides.

IgE.C ϵ mX.migis, which is IgE with C ϵ mX.migis at its C-terminus; it is identical to the extracellular domain portion of mIgE but lacks the transmembrane and intracellular regions so that it can be secreted; and (3) IgE.C ϵ mX, which is IgE with the C ϵ mX sequence at the C-terminus. Because expression levels of these recombinant proteins were very low, they were not purified. Instead, the conditioned media were used in the binding study. The relative expression levels of the three proteins in conditioned supernatants from 293T transfectants were compared by using antigen (EphA2) as a capture reagent and anti-IgE Fc specific antibody as the detector reagent. We showed that although sIgE and IgE.C ϵ mX proteins were expressed at much higher levels than IgE.C ϵ mX.migis (Fig. 6A), D5 and F4 IgG bound only IgE.C ϵ mX.migis and not sIgE and IgE.C ϵ mX proteins (Fig. 6B). These results together with the result of Fig. 5 indicated that D5 and F4 antibodies bind IgE only when IgE has both C ϵ mX and ϵ -migis portions at its C-terminus.

3.5. Antibody F4, but not D5, cross react with cynomolgous C ϵ mX.migis

Since part of the c ϵ mX region is an integral part of the epitope of F4 and D5 antibodies and since c ϵ mX is present only in primates (Chen et al., 2010) we investigated whether D5 and F4 can bind to cyC ϵ mX.migis as a way to determine if they could be studied in cynomolgous monkeys for future pre-clinical studies. Comparison of the C ϵ mX.migis peptide from human and cynomolgous monkey showed that there is 78% identity between the two. As shown in Fig. 7, in an ELISA assay only F4 IgG bound to cyC ϵ mX.migis peptide to the same extent as to huC ϵ mX.migis. In contrast, D5 IgG showed

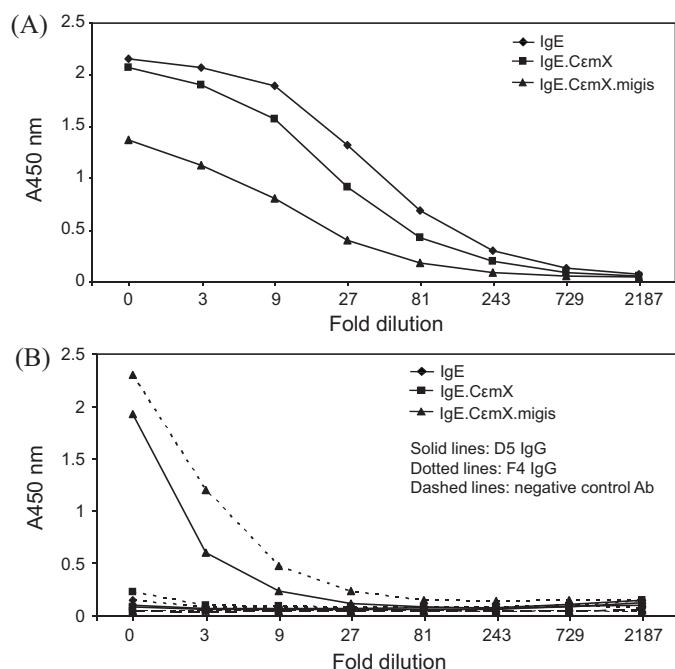


Fig. 6. ELISA experiment showed that D5 and F4 IgG bound specifically to IgE.CεmX.migis. (A) Expression analysis of recombinant IgE, IgE.CεmX, and IgE.CεmX.migis produced by mammalian 293T cells. Cell supernatants were captured onto 96-well ELISA plates coated with EphA2 antigen. The binding of recombinant proteins (with antigen-binding specificity for EphA2) was detected with goat anti-human IgE Fc–HRP conjugate. As shown, IgE.CεmX.migis protein was expressed at a much lower level than the other two. (B) Binding analysis of D5 and F4 IgG. ELISA plates were prepared as described above to capture recombinant proteins from supernatants. D5 and F4 IgGs were then titrated on separate plates, and bound D5 and F4 IgG was detected by anti-human IgG Fc–HRP conjugate. Solid line: D5 IgG; dotted line: F4 IgG and dashed line: isotype control IgG. The data showed that both D5 and F4 IgG bound only to the recombinant protein that has both CεmX and ε-migis region.

same level of binding to huCεmX.migis as F4 but showed trace amount of binding to cyCεmX.migis only at high concentration.

3.6. Antibodies D5 and F4 elicit ADCC on mIgE-expressing cells

To test whether D5 and F4 IgG1-3M can lyse the mIgE-cells via antibody-dependent cellular cytotoxicity, ADCC assays were carried out in 293T and NS0 cells stably co-transfected with mIgE, CD79a, and CD79b. The variable domain for the mIgE was cloned from a monoclonal antibody that binds to the F-protein of respiratory syncytial virus, an antigen not present on 293T and NS0 cells. As shown in Fig. 8A, D5 IgG1-3M induced ADCC only in the line that

expressed mIgE. The ADCC activity on the mIgE-positive cell line could be inhibited when D5 IgG1-3M was pre-incubated with the CεmX.migis peptide. These results showed that D5 IgG1-3M could specifically bind to its target on cells and induced cytotoxicity through ADCC. The ADCC result of F4 IgG1-3M on mIgE displaying 293T cells was similar to D5 IgG1-3M (data not shown). We also tested the ADCC activity of D5 and F4 IgG1-3M in a dose dependent manner on NS0 cells stably expressing mIgE, CD73a and CD73b. As shown in Fig. 8B both antibodies showed comparable dose-dependent ADCC activity similar to that seen on the mIgE displaying 293T cells. Thus ADCC activity was evident on 2 different target cell lines.

3.7. Antibody F4 reduces IgE expressing B cells differentiated in vitro from PBMC of healthy donors

To test whether anti-CεmX.migis antibody, F4 IgG1-3M can elicit an effect on human IgE expressing B cell, an ELISPOT assay was done using PBMCs that had been isotype switched to IgE expressing cells in vitro. This was done because we did not have access to samples from allergic patients or patients with high IgE levels (e.g. hyper IgE syndrome) from which IgE⁺ B cells could be directly isolated. As shown in Fig. 9 F4 IgG1-3M showed marked reduction in the number of IgE positive spots in an ELISPOT assay compared to an isotype control. The number of IgE positive spots in the control IgG sample varied among different donors from about 10 to more than 60 and in each case F4 IgG1-3M showed about a log reduction in the number of spots.

4. Discussion

A monoclonal antibody that depletes IgE-producing B cells might treat allergies by eliminating IgE production while leaving B cells of other antibody isotypes unharmed. Our interest in developing such an antibody was influenced by the success of the antibody omalizumab. Omalizumab is an FDA-approved humanized anti-IgE antibody that binds IgE and inhibits its binding to Fcε receptors (IgεRs). Consequently, IgεRs on mast cells and basophils are downregulated (MacGlashan et al., 1997; Rambasek et al., 2004; Belliveau, 2005). Although omalizumab has been effective as an add-on allergy therapy, because of dosing restrictions and because its primary mechanism is to neutralize soluble IgE, omalizumab therapy is not practical for patients with heavy body weight or elevated IgE blood levels (Rambasek et al., 2004). Chang and colleagues have identified murine monoclonal antibodies against ε-migis and CεmX region of human IgE by hybridoma approach (Chang et al., 1990; Chen et al., 2002) and have proposed their therapeutic application for IgE-mediated allergic diseases. Here we explored a phage display approach coupled with extensive screening that involved live cell FACS assays for identifying human antibodies specific to IgE-producing B cells to develop for the same application. Two target regions were investigated. They were ε-migis and CεmX.migis junction.

Our results showed that panning and screening of phage human antibody library against ε-migis peptide did not yield antibodies specific to mIgE-displaying cells. This could be due to high sequence homology of ε-migis with a peptide within ARAP3 protein (Figs. 2B and 5). Perhaps, if we revise our panning and screening strategy by using ARAP3 peptide to absorb the library before and during panning against ε-migis, we may be able to steer the selection toward the epitopes that are specific to ε-migis. However, we did not pursue this target further in this study.

In the effort to target CεmX.migis junction region, a peptide containing the last 8 amino acids of CεmX followed by the 15-residue ε-migis was designed, synthesized and used for panning the phage library. After panning and screening, we identified 108 clones that

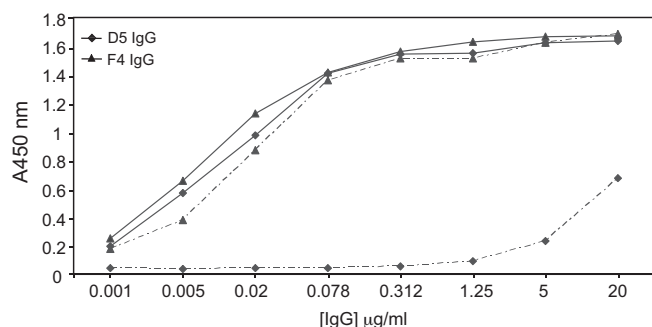


Fig. 7. Cross reactivity analysis of D5 and F4 IgG to human and cynomolgous CεmX.migis peptides. The experiment was done as described in section 2.7. Solid line: human CεmX.migis; dotted line; cynomolgous CεmX.migis. The results show that F4 but not D5 IgG cross react with cynomolgous CεmX.migis peptide.

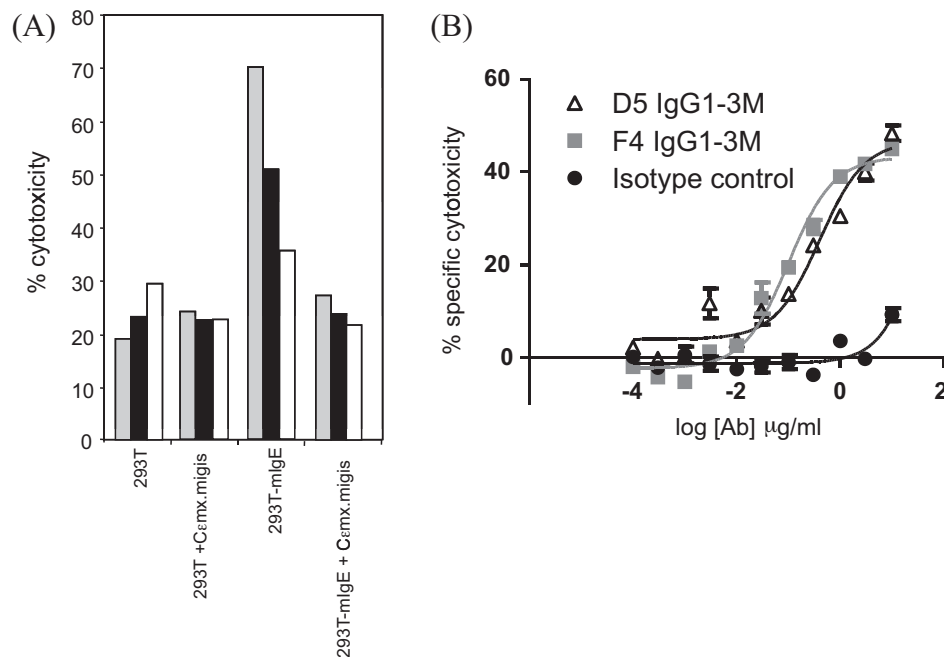


Fig. 8. (A) ADCC activity of anti-Cεmx.migis antibody D5 as determined by LDC release. D5 IgG1-3M induced ADCC to 293T cells that express mIgE, CD79a, and CD79b on the surface. D5 was tested at 3 different concentrations, 100 μg/ml (gray); 10 μg/ml (black); 1 μg/ml (white). ADCC on mIgE-expressing 293T cells was significantly higher than on the parental 293T cells (background). Background ADCC activity on parental 293T cells was unaffected by pre-incubation of the antibody with its cognate antigen, Cεmx.migis peptide. But on the mIgE-expressing 293T line, the ADCC activity could be inhibited by pre-incubation of the antibody with Cεmx.migis peptide. A negative control IgG had no difference in ADCC activity between parental and mIgE-expressing 293T lines (data not shown). (B) ADCC activity of D5 and F4 IgG1-3M was determined on NS0 cell line stably expressing mIgE, CD79a, and CD79b using an LDH release assay. Both anti-Cεmx.migis antibodies demonstrated substantial ADCC activity in a dose dependent manner. All ADCC assays were done in triplicate. Results are mean ± standard deviation and representative of two independent experiments.

bound specifically to Cεmx.migis peptide. We found an additional screening effort was required to find the antibodies that can bind mIgE-expressing cells exclusively. We set up an ELISA screening assay involving competition by A1c (anti-ε-migis antibody binding to all cells tested) and B1 (representing the anti-Cεmx.migis antibodies that did not bind any cells). This screen resulted in the isolation of 2 clones, D5 and F4 which specifically bound to mIgE-expressing cells (Fig. 4). This illustrates the potential importance of competition-based screening for identifying rare and unique clones within a large population of panned phages falling short of desirable characteristics. D5 and F4 could induce cell killing by ADCC (Fig. 8). Two different target cells used in this study are engineered cell lines expressing mIgE along with CD79a and CD79b, much like the BCRs of mIgE positive memory B cells. Because D5 and F4 can access their epitope on mIgE in the presence of CD79a and CD79b we think that they will also be able to target mIgE positive primary B cells. To confirm this assumption we evaluated whether F4 IgG1-3M can inhibit growth and/or secretion of IgE from primary human B cells that were isotypically switched in vitro to produce IgE. As shown in Fig. 9, compared to an isotype control antibody, F4 significantly reduced the number of IgE positive spots in the ELISpot assay. This approach was taken because we did not have access to patient samples with high number of IgE⁺ B cells. Only F4 was used for this study for the following two reasons: (i) the efficiency of IgE class switching obtained in vitro is low and allowed us to study only one antibody in greater detail and (ii) F4 is strongly cross reactive with cyCεmx.migis and therefore of interest for further evaluation and development. The ability of F4 to act on IgE⁺ human B cells proves that it can bind to its target not only on engineered cell lines but also on primary cells. We do not, however, know at this time whether the mechanism of action of F4 on the human IgE⁺ B cells is the depletion of the cells or inhibition of IgE synthesis or both.

Recently, there were reports on identification and/or characterization of antibodies targeting Cεmx and ε-migis. Chen et al. (2010) reported on mouse antibodies against the N-terminal and middle portion of human Cεmx that inhibited IgE synthesis in PBMCs from atopic dermatitis patients. This report also described an antibody, a20, against the C-terminal part of Cεmx which failed to bind to mIgE in the context of a BCR complex. The other report by Brightbill et al. (2010) demonstrated the efficacy of a humanized anti-ε-migis antibody (47H4) in a human ε-migis transgenic mouse model. This antibody showed specific binding to engineered Daudi cells expressing ε-migis. It is possible that the epitope for 47H4, which is derived by hybridoma technology, is different from our phage-derived A1c antibody. The work presented in this manuscript shows that human antibodies, D5 and F4 directed towards the Cεmx.migis junction can bind specifically to cells expressing huIgE BCRs and induce ADCC to them and that F4 act directly on human IgE⁺ B cells. Genetic polymorphisms have been reported in the N-terminal part of Cεmx region (Wan et al., 2010). Since D5 and F4 bind to the junction of Cεmx.migis and have been shown to require only the last 8 amino acids of Cεmx and the 15 amino acid migis we think that the binding of these two antibodies to mIgE will be unaffected by Cεmx polymorphisms. In addition, both D5 and F4 are fully human antibodies. Because D5 and F4 are fully human antibodies that can bind to IgE BCRs and elicit inhibitory activities and because they are directed at regions that are not known to have genetic polymorphisms they are attractive candidates for further investigation. These antibodies, as well as antibodies described by Chen and Brightbill, may have therapeutic potential to reduce IgE levels in allergic patients. However, there is recent evidence for the existence of long-lived, antigen-specific IgE-secreting plasma cells in mouse after aerosol immunization and these cells likely do not have mIgE (Luger et al., 2009). This suggests that the strategy of targeting Cεmx.migis would be most useful to eliminate mIgE⁺

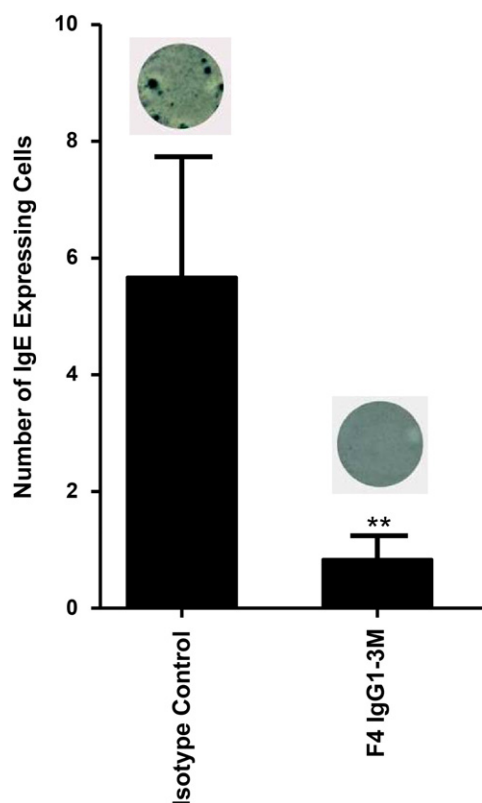


Fig. 9. Effect of F4 IgG1-3M on primary B cell isotype switched in vitro from normal human PBMC to express IgE. Several different donor samples have been studied. There is donor to donor variation in the number of B cells that can be isotype switched to IgE. In every case F4 IgG1-3M IgG significantly reduced the number of IgE B cells compared to isotype control. In the experiment represented here the IgE spots are relatively few but more conspicuous compared to other donors.

memory B cells. It remains to be determined whether or not IgE-secreting plasma cells play a key role in the pathology of some or all IgE-induced allergic reactions. Their role may have an impact to the therapeutic potential of the mIgE-B cell-targeting therapy.

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Corrigendum

Corrigendum to “Targeting the junction of C ϵ mX and ϵ -migis for the specific depletion of mIgE-expressing B cells”

[Mol. Immunol. 52 (October–December (3–4)) (2012) 279–288]

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The second last line under Section 2.1 should read “Of note, peptides 4, 5, and 7 were not soluble in PBS”, instead of “Of note, peptides 3, 4, and 6 were not soluble in PBS”.

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