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AllergoOncology: Expression platform development and functional profiling of an anti-HER2 IgE antibody

To the Editor,

Monoclonal antibodies approved for the treatment of cancer belong to the IgG class (most often IgG1). However, IgG has limited tissue half-life (2-3 days), relatively low affinity for cognate Fc receptors and the disadvantage of interaction with inhibitory Fcγ receptors, abundant in the tumour microenvironment. Conversely, IgE class antibodies may offer new options for cancer therapy, based on high affinity for cognate Fcε receptors expressed on different, often tumour-resident, immune effector cells such as macrophages and mast cells, and lack of inhibitory Fc receptors.[1] IgE-mediated tissue surveillance functions known to potentiate "allergic" or "pathogen/parasite-clearing" immunity could be re-directed against tissue-resident tumours.[2,3] IgE antibodies recognizing the tumour-associated antigen folate receptor α (FRα) induced superior immune responses in disparate in vivo models, highlighting potential opportunities for FRα-expressing ovarian carcinomas.[2] In breast cancer, in vitro studies of trastuzumab (IgG1) and an engineered trastuzumab IgE recognizing the tumour-associated antigen HER2/*neu* indicated that IgE could complement or possibly improve the clinical performance of trastuzumab.[4] The first-in-class IgE antibody (MOv18) is undergoing an early phase clinical trial in patients with FRα-expressing carcinomas (NCT02546921, www.clinicaltrials.gov).

Despite considerable progress, production of monoclonal antibodies remains time-consuming and labour-intensive. One reason is the requirement for expression of heavy (HC) and light chains (LC) in a controlled manner, usually cloned in separate expression vectors using enzymatic restriction digestion and ligation. This introduces experimental variability in expression procedures and is often inefficient. These limitations also concern the study of anti-allergen IgE, where Fabs rather than full-length antibodies are commonly expressed and evaluated.[5-7] Therefore, antibody cloning systems are moving towards utilization of single dual-expression plasmids (eg pcDNA3.3 and pVitro1 hygro-mcs), to increase antibody production.[8]

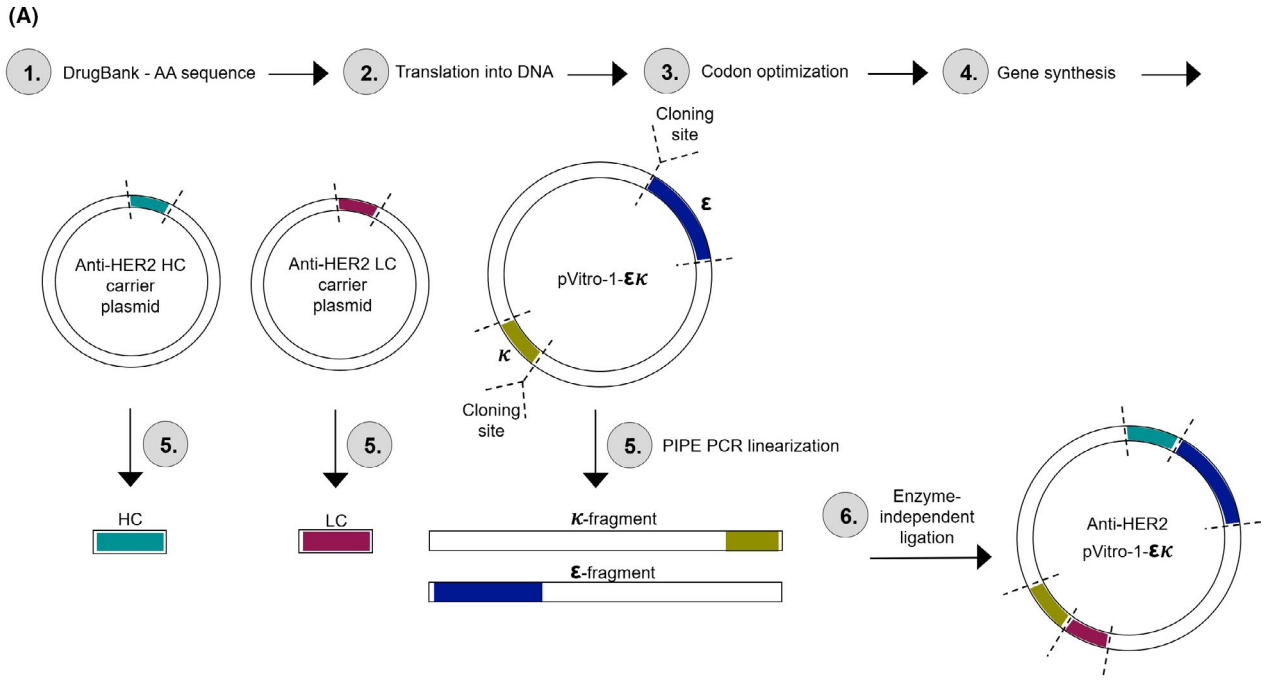
Building upon ours and others' previous methodologies, we report the efficient transient expression and functional evaluation of IgE, exemplified using the variable region sequences of trastuzumab and human IgE constant regions (anti-HER2 IgE).

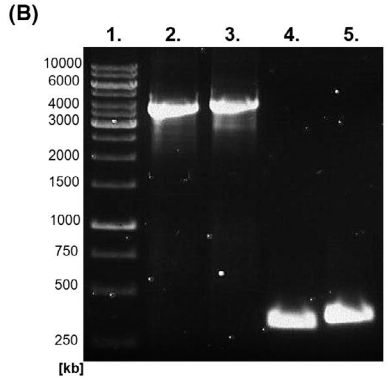
We employed polymerase incomplete primer extension (PIPE) PCR cloning and enzyme-free assembly of DNA fragments. The amino acid sequences of trastuzumab variable light (VL) and heavy (VH) chain regions were manually codon-optimized for a human expression host and cloned into a pVitro1-hygro-mcs dual-expression vector containing precloned cassettes of the human epsilon HC and kappa LC using PIPE PCR cloning methodology (Figure 1A).[8] PIPE PCR was performed using the pVitro1 plasmid to generate linear PCR fragments with 5′ PIPE overhangs, and trastuzumab variable region fragments to derive VL and VH region fragments with 5′ PIPE overhangs (DNA fragment sizes by agarose gel electrophoresis, Figure 1B).

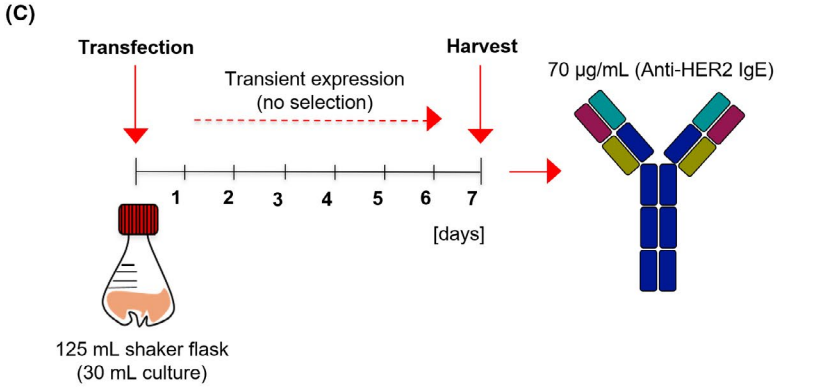
Expression was conducted transiently in human embryonic kidney (Expi293F) cells without antibiotic selection, in 30 mL serum-free suspension cultures (Figure 1C). Variable region codon optimization enhanced antibody yields (~7-fold; Figure 1D). Peak antibody concentrations (70-80 μg/mL) were achieved within 7-9 days (supernatants harvested after 7 days, Figure 1C,E). After purification, total yields were 60 μg/mL (> 85% purification efficiencies; Figure 1F). SDS-PAGE of purified antibodies under nonreducing conditions showed a 250 kDa band, likely reflecting high antibody glycosylation), and reducing conditions revealed two signals (75 kDa [HC], 25 kDa [LC]), and a slight signal (100 kDa) likely representing different HC glycoforms (Figure 1G). HPLC analysis demonstrated assembly of monomeric IgE (Figure 1H).

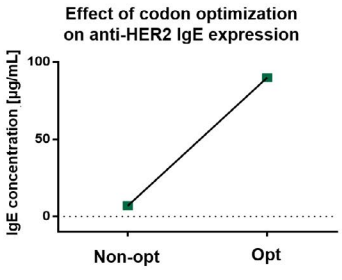
Like trastuzumab, anti-HER2 IgE recognized HER2/*neu*-overexpressing (BT-474, ZR75-30) breast cancer cells and moderately expressing MCF-10 normal breast cells, and its HER2 antigen recognition kinetic profile on tumour cells was comparable to trastuzumab (Figure 2A). Anti-HER2 IgE and trastuzumab similarly restricted breast cancer cell viability and epidermal growth factor signalling, while addition of antibodies together did not improve HER2 signalling inhibition (Figure 2B,C). Consistent with FcεR-binding MOv18 IgE[1,2] (produced in SP2/0 cells), anti-HER2 IgE recognized RBL SX-38 rat basophilic leukaemia cells, expressing the human tetrameric FcεRI($\alpha\beta\gamma_2$), and human U937 monocytes expressing the low-affinity IgE receptor FcεRII/CD23 upon IL-4 stimulation (Figure 2D). Similar to MOv18 IgE, anti-HER2 IgE recognized FcεRI-expressing human primary monocytes and anti-HER2 IgE binding kinetics to RBL SX-38 were comparable to those of MOv18 IgE (Figure 2).

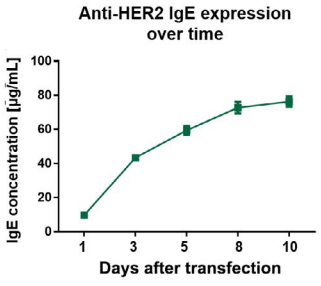
Anti-HER2 IgE induced >2-fold higher ADCC of HER2-overexpressing breast cancer cells by unstimulated and IL-4

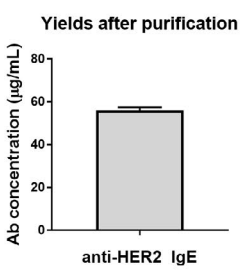

(A)
1. DrugBank - AA sequence
2. Translation into DNA
3. Codon optimization
4. Gene synthesis
Anti-HER2 HC carrier plasmid
Anti-HER2 LC carrier plasmid
pVitro-1-εκ
Cloning site
ε
κ
Cloning site
5.
5.
5. PIPE PCR linearization
HC
LC
κ-fragment
ε-fragment
6. Enzyme-independent ligation
Anti-HER2 pVitro-1-εκ

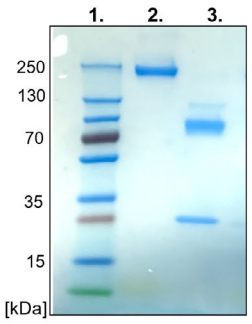

(B)
1. 2. 3. 4. 5.
10000
6000
4000
3000
2000
1500
1000
750
500
250
[kb]


(C)
Transfection
Harvest
Transient expression (no selection)
1 2 3 4 5 6 7
[days]
125 mL shaker flask (30 mL culture)
70 µg/mL (Anti-HER2 IgE)


(D)
Effect of codon optimization on anti-HER2 IgE expression
IgE concentration [µg/mL]
100
50
0
Non-opt
Opt


(E)
Anti-HER2 IgE expression over time
IgE concentration [µg/mL]
100
80
60
40
20
0
1 3 5 8 10
Days after transfection


(F)
Yields after purification
Ab concentration (µg/mL)
80
60
40
20
0
anti-HER2 IgE


(G)
1. 2. 3.
250
130
70
35
15
[kDa]

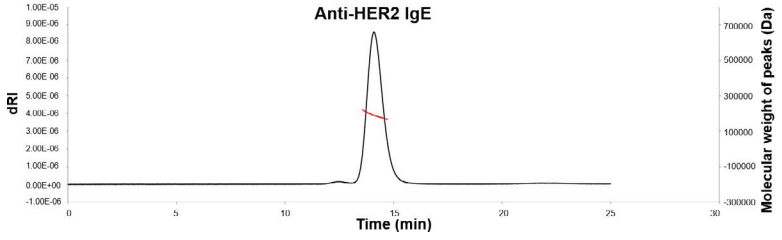

(H)
Anti-HER2 IgE
dRI
Molecular weight of peaks (Da)
Time (min)

FIGURE 1 Anti-HER2 IgE cloning and generation. A, Cloning strategy. 1-4: Variable region DNA sequence generation. 5: Trastuzumab variable region plasmids, pVitro1 plasmid with kappa/epsilon constant chains linearized (PIPE PCR), generating 4 fragments with 5′ PIPE overhangs. 6: Linear fragments assembled nonenzymatically (pVitro-1-$\varepsilon\kappa$). B, Agarose gel electrophoresis (PIPE fragments). 1: DNA ladder, 2: ε-fragment (4099 bp), 3: κ-fragment (4119 bp), 4: LC (364 bp), 5: HC (408 bp). C, Expression strategy. D, 7-day yields following codon optimization (representative). Expression before (E) and after (F) purification (±SD, representative of n = 2). G, SDS-PAGE: 1: protein standard, 2: nonreducing, 3: reducing conditions. H, HPLC trace after size exclusion chromatography

stimulated U937 effector cells compared with isotype controls (Figure 2E). Anti-HER2 IgE triggered higher ADCC against breast cancer cells by peripheral blood mononuclear cells (PBMCs from human volunteers, HV, Figure 2E) and >-fold higher ADCC by RBL SX-38 cells (Figure 2F) compared with isotype controls (see Appendix S1).

Anti-HER2 IgE induced degranulation of RBL SX-38 cells when cross-linked by polyclonal anti-IgE on the cell surface (left) or by

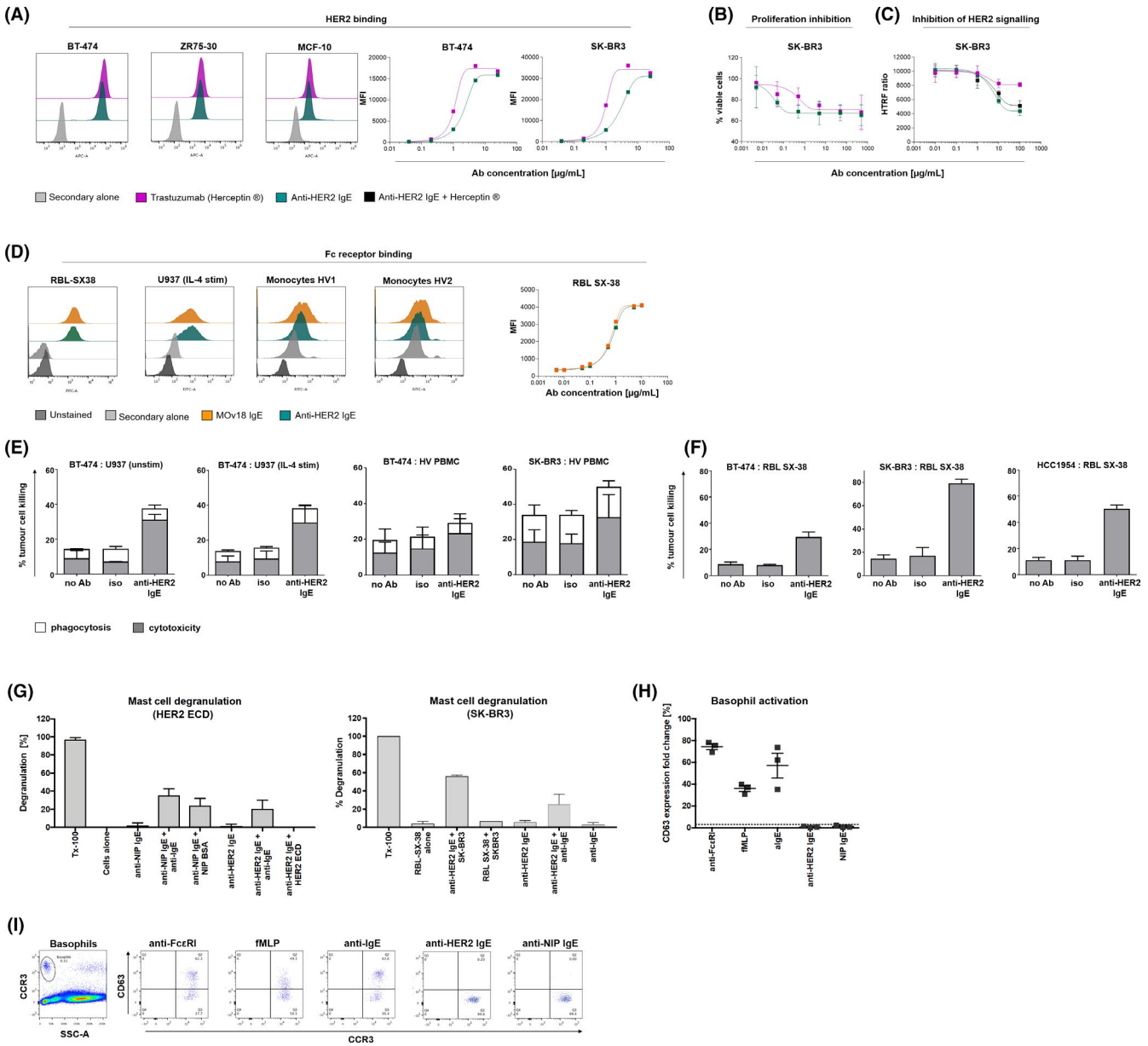


FIGURE 2 Anti-HER2 IgE functional characterization. A, Flow cytometric binding/kinetic profiles to breast cancer and normal breast (MCF-10) cells. IgE reduced breast cancer cell viability (B), and HER2/*neu* signalling (n = 2) (C). D, Flow cytometric binding/kinetic profiles of IgE to human FcεR-expressing: RBL SX-38 mast cells, U937 monocytes, human monocytes (healthy volunteers, HV; anti-FRα IgE [MOv18]: control). E, F, IgE-mediated % tumour cell killing (±SD): (E) by U937 (n = 3), human (HV) PBMC (n = 6); (F) by RBL SX-38. G, RBL SX-38 degranulation experiments (β-hexosaminidase release, Triton X-100 lysis (Tx100): 100% granule release, representative of n = 2). H, I, Anti-HER2 IgE stimulation in basophil activation test (BAT) (G), and representative flow cytometric dot plots (I), depicting lack of basophil activation with anti-HER2 IgE stimulation

HER2-expressing tumour cells (right), but not without cross-linking stimulus or with recombinant monomeric antigen (HER2 ectodomain [ECD]; Figure 2G). In basophil activation tests (BAT) conducted in unfractionated human blood, anti-HER2 IgE did not induce basophil activation, monitored by upregulation of the activation marker CD63 (Figure 2H,I). Mast cell and basophil tests therefore confirm lack of activation with IgE in the absence of cross-linking stimuli,[9] supporting potential safe administration in human circulation.

IgE immunotherapy may offer a promising approach for cancer treatment, contributing to the emerging field of AllergoOncology, focused on dissecting interplay between IgE, allergy and malignancy. The development of efficient platforms for speedy generation of full-length IgE at appreciable yields for numerous evaluations to expedite the field remains challenging. Our herein-described multi-gene cloning, enzyme-free assembly system for rapid expression of functionally active antibody, within 7-9 days from transfection to purification in serum-free cultures (2 mg purified material from 30 mL), readily established even in "small" environments, surpassing previous platforms in expression efficiency, speed (7-9 days vs 4-6 weeks) and yields (70-80 mg/mL vs <20-25 mg/mL),[4] meets these challenges. IgE maintained Fab- and Fc-mediated properties, including antigen and receptor binding, ADCC and degranulation, contributing to the most important/prominent antibody functionalities. These suggest that under conditions akin to those of tumours, when encountering high levels of HER2-expressing cancer cells, anti-HER2 IgE may trigger mast cell activation and antitumour effector functions. Importantly, the lack of anti-HER2 IgE blood basophil activation points to diminishing potential safety concerns associated with using IgE class antibodies in cancer immunotherapy. Our report of transient cloning and rapid antibody production greatly facilitates the study of IgE structural and immune functional attributes and may find numerous applications in allergy, biotechnology and immunology-related fields.

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CONFLICTS OF INTEREST

SN Karagiannis and JF Spicer are founders and shareholders of IGEM Therapeutics Ltd. SN Karagiannis holds a patent on antitumour IgE antibodies. All other authors have declared that no conflict of interest exists.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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The impact of prescribed fire versus wildfire on the immune and cardiovascular systems of children

To the Editor

The increase in wildfires associated with climate change augments the impact of air pollution on health in many areas of the country. When wildfires occur, there is an increase in asthma attacks and associated comorbidities,[1,2] especially for asthma hospitalization in ages 0-5 years[3] and more recently, it has been shown that there are increases in cardiovascular events.[4] Given the health risks associated with high-intensity wildfires, there is motivation to increase the use of lower intensity prescribed fires. Prescribed burns decrease the buildup of flammable vegetation and subsequent fuel for wildfires, mitigating the spread and intensity of wildfires. However, prescribed fire raises public concerns because of the additional pollutant exposure.

Therefore, our objective is to determine whether there are differential health consequences with a prescribed fire vs wildfire. We focus on children given their reduced lung size, increased metabolic rates, higher respiratory rate, and developing immune systems,[5] and because in macaque monkeys who are exposed to wildfire smoke in infancy, there is associated immune dysregulation and decreased lung function in adolescence.[6] We hypothesize that the health impacts of a prescribed fire are less detrimental to the respiratory and cardiovascular systems than a wildfire in school-aged children and that T-cell skewing and epigenetic modulation will occur with exposure to wildfire more than from exposure to a prescribed fire.

We analyzed data collected from a convenience sample of subjects (n = 220) over a period of 2 years living in Fresno, CA, all of whom were potentially exposed to smoke from fires, which consisted of similar varieties of coniferous trees, in nearby Yosemite National Park. Health questionnaires, blood samples, and vital signs were collected, and subjects were selected that had their blood drawn 3 months after a prescribed fire or wildfire, because our prior research indicates that this time frame is associated with increased methylation of the Foxp3 gene.[7] Using this criteria, we analyzed data from 32 children (median age = 7 [range 7; 8] yrs, 38% asthmatic as per NHLBI guidelines) exposed to a prescribed fire 70 miles away covering 553 acres in March, 2015, and 36 children (median age = 8 [range 7; 8] yrs, 25% asthmatic) exposed to a wildfire 70 miles away covering 415 acres in September 2015. A control group of 18 children was also compared (median age = 8 [range 7; 8] yrs; 21% asthmatic), who had no obvious exposure to wildfires or prescribed fire and were living in the San Francisco Bay area, where pollution levels