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ORIGINAL ARTICLE

Using the allergic immune system to target cancer: activity of IgE antibodies specific for human CD20 and MUC1

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Abstract Monoclonal antibodies are widely used in the treatment of many B cell lymphomas and certain solid tumors. All currently approved therapeutic monoclonal antibodies are of the immunoglobulin G (IgG) isotype. We hypothesized that tumor-specific monoclonal antibodies of the IgE isotype may serve as effective cancer therapeutics. To test this hypothesis, we produced mouse–human chimeric IgE antibodies specific for the human B cell antigen CD20 and the epithelial antigen MUC1. We demonstrate here that anti-hCD20 IgE antibodies have in vitro cytotoxic activity when used with purified allergic effector cells derived from umbilical cord blood. At an effector-tumor ratio of 2:1, mast cells and tumor-specific IgE induced a 2.5-fold increase in tumor cell death, as compared to control IgE. Similar results were observed when eosinophils were used as effector cells. In an in vivo murine model of breast carcinoma, administration of anti-hMUC1 IgE reduced the growth of MUC1$^+$ tumors by 25–30 % in hFcεRI transgenic mice. In contrast, local production of IgE and cytokines chemotactic for macrophages, eosinophils and mast cells led to complete tumor eradication. These results suggest that allergic effector cells activated by IgE and cell surface antigens have the capacity to induce tumor cell death in vitro and in vivo. The use of chimeric antibodies and hFcεRI transgenic mice will greatly enhance investigations in the nascent field of allergo-oncology.

Keywords Tumor immunity · Antibodies · Mast cells · Eosinophils

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ANOVA	Analysis of variance
CBEos	Cord blood-derived eosinophils
CBMC	Cord blood-derived mast cell
CFSE	Carboxyfluorescein diacetate succinimidyl ester
E:T	Effector to target ratio
FcεRI	Fragment crystallizable epsilon receptor I
hCD20	Human CD20
hMUC1	Human mucin 1
hSCF	Human stem cell factor
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
mAb	Monoclonal antibody
MAHA	Mouse anti-human antibody
PBMC	Peripheral blood mononuclear cell
PI	Propidium iodide
SAM	Significance analysis of microarrays
s.c.	Subcutaneous

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SCID	Severe combined immunodeficiency
SD	Standard deviation
TRAIL	TNF-related apoptosis inducing ligand
TNF	Tumor necrosis factor

Introduction

Monoclonal antibodies (mAbs) specific for cell surface tumor antigens are valuable therapeutic tools in the treatment of cancer. The high specificity and low toxicity of antibodies make them attractive therapeutic agents. To develop the next generation of antibody products, research is increasingly focused on modifying the antibody constant region (Fc region). These efforts can be broadly divided into two categories. The first approach attempts to conjugate toxic compounds to the Fc region, such as radiation sources or bacterial toxins [1]. The other approach is to modify the antibody Fc region to increase the binding affinity for its receptor, leading to more pronounced Fc receptor-mediated effector functions [2–4].

One strategy that has not been fully explored is the potential contribution of different antibody isotypes to observed anti-tumor effects. Nearly all monoclonal antibodies (mAbs) approved for use in humans are of the gamma-1 isotype. The contribution of other isotypes to anti-tumor responses has been explored in vitro. One report by Bruggeman et al. constructed cognate antibodies of different isotypes and tested their ability to lyse target cells via complement or antibody-dependent cytotoxicity (ADCC) [5]. These investigators used peripheral blood mononuclear cells (PBMCs) as effector cells. Their data demonstrated the superiority of IgG1 antibodies in both assays. However, the use of PBMCs may have underestimated the role of other cell types either absent or not well represented in the peripheral blood mononuclear fraction.

Herein, we test the hypothesis that other antibody isotypes might demonstrate anti-tumor efficacy. Specifically, antibodies of the epsilon isotype, with their ability to activate mast cells, basophils, eosinophils and dendritic cells, might be effective anti-cancer agents. Indeed, Karagiannis et al. have demonstrated that tumor-specific IgE antibodies can inhibit ovarian cancer growth in SCID mice when co-administered intra-abdominally with human PBMCs [6, 7]. In their model, IgE antibodies specific for the folate binding receptor expressed on the surface of ovarian tumors were more effective in slowing tumor growth than the cognate IgG mAb. Serum IgE generated against HER2 mimetopes was also shown to be protective in a murine model of transplantable HER2$^+$ breast cancer [8].

To more fully test these ideas, we produced and purified mouse–human chimeric IgE monoclonal antibodies specific for cell surface antigens found on common human tumors. Our goal was to design a small panel of chimeric IgE antibodies and then test their ability to mediate tumor lysis in vitro using purified populations of effector cells. The data demonstrated that tumor-specific IgE antibodies, along with purified populations of allergic effector cells, could indeed mediate tumor cell death in vitro. There appeared to be an antibody-dependent and independent mechanism involved. The cytotoxic effects of mast cells were partially due to tumor necrosis factor (TNF), while those of eosinophils were caused by cationic factors. When we tested the ability of IgE antibodies to mediate tumor rejection in a murine model, we found that they had modest activity when administered for a brief period early in tumor growth. In contrast, more striking results were obtained when the IgE was continually produced in the tumor microenvironment, together with chemokines that attract allergic effector cells. These data demonstrate that both tumor-specific IgE antibodies and allergic effector cells must be present in the microenvironment before meaningful anti-tumor responses can be observed.

Materials and methods

Cell lines and reagents

The VU-3C6 hybridoma was provided by Dr O. Finn (University of Pittsburgh, Pittsburgh, PA). OCI-Ly8 lymphoma was a kind gift from R. Levy (Stanford University, Stanford, CA). Human PBMCs were obtained from Ficoll-Paque Plus (Stem Cell Technologies, Vancouver, BC, Canada) separation of whole blood. All other cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). 4T1.hMUC1 and A20.hCD20 cells were created by transfection of the hMUC1 and hCD20 cDNA into 4T1 and A20 cells, respectively. Recombinant human stem cell factor (rhSCF) and rhIL6 were gifts from Amgen (Thousand Oaks, CA). All other cytokines were purchased from Peprotech Inc (Rocky Hill, NJ). StemSpan® H3000 media was purchased from Stem Cell Technologies (Vancouver, Canada). BDCell Quantum Yield (QY) and Animal Component Free (ACF) media were purchased from Becton–Dickinson (Franklin Lakes, NJ). Pluronic F-68 Solution was purchased from Sigma Aldrich (St. Louis, MO). All other media and media supplements were from Invitrogen Corp (Carlsbad, CA). Anti-cytokine antibodies were from eBioscience (San Diego, CA). All other antibodies were purchased from Becton–Dickinson (BD, Franklin Lakes, NJ), unless otherwise stated.

Amplification of V_H,V_L and constant region genes by polymerase chain reaction (PCR)

Cytoplasmic RNA was isolated from 1F5 and 3C6 hybridomas, SKO myeloma and human PBMCs (RNeasy Mini Kit,

Qiagen GmbH, Hilden, Germany), followed by first-strand cDNA synthesis (SuperScript RT system, Invitrogen). Murine V_H and V_L genes were amplified by PCR using a set of consensus leader sequences and constant region sequence-specific primers (Supplementary Table 1a). All primers were tested to identify those that were specific for the genes encoding each antibody heavy and light chain. KpnI or HindIII sites were incorporated on both ends of the primers, and a Kozak sequence was engineered into the 5′ end of the variable region genes. The human kappa light chain gene was amplified from PBMC cDNA and the hIgE constant region gene from SKO cDNA. XbaI sites were inserted on both ends of each constant gene region. All primers used are shown in Supplementary Table 1b.

Plasmids and transfection

pcDNA 3.1(+) plasmids with neomycin, zeocin, and hygromycin selection markers, pEF6 plasmid with a blasticidin selection marker, and T4 DNA ligase, were purchased from Invitrogen. pkappa and pEpsilon plasmids were constructed by inserting the human kappa and hIgE constant region genes into the XbaI site of pcDNA 3.1(+) or pEF6 plasmids. Complete light and heavy chain plasmids were constructed for each chimeric IgE antibody by inserting the variable region genes into the HindIII site of pkappa, or the KpnI site of pEpsilon. The final plasmids are described in Fig. 1. The entire process was repeated with the mouse kappa and epsilon constant region genes to create the heavy and light chain vectors for 3C6.mIgE.

CHO-K1 cells were transfected with an equimolar mixture of all four plasmids (pkappaI and II, pEpsilonI and II), using the FuGENE® transfection reagent (Roche Pharmaceuticals, Nutley, NJ). After 24 h, the cells were washed, and fresh media containing neomycin (500 ug/ml), zeocin (300 ug/ml), blasticidin (4 ug/ml) and hygromycin (300 ug/ml) was added. Cells were screened for IgE production via ELISA and cloned by limiting dilution.

ELISAs

Capture antibodies or antigens were coated on flat-bottom 96-well Nunc Maxisorp plates (Nalgene Nunc, Rochester, NY) in 0.1 M sodium carbonate buffer (pH 9.6) overnight at 4 °C. Plates were washed with PBS 0.05 % Tween-20 containing 2 mM EDTA (PBST). Plates were then blocked with 1 % BSA PBST at room temperature, washed, and probed with detection antibodies. ELISA antibodies and

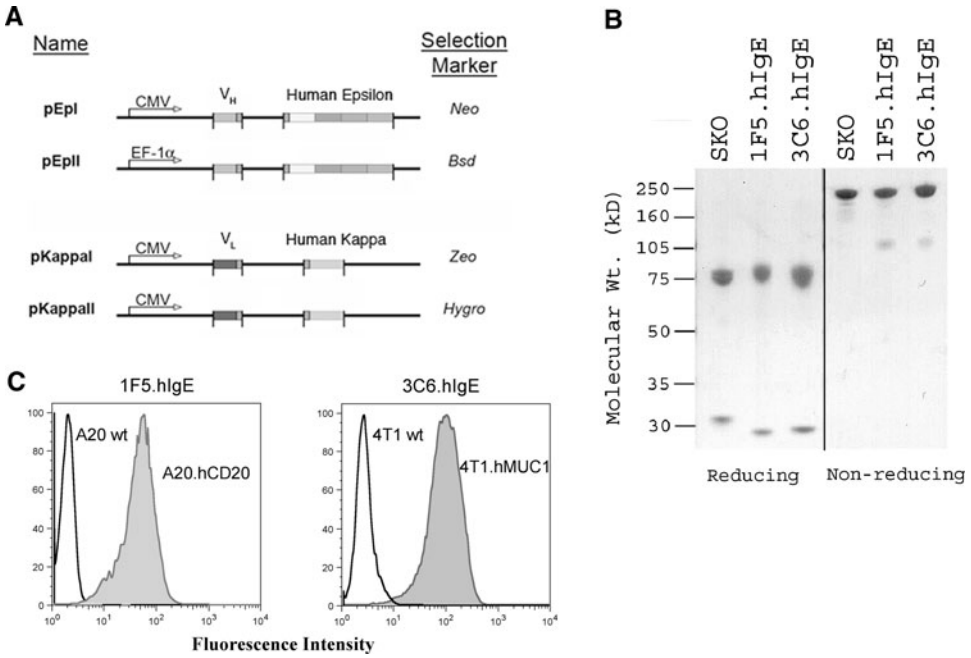

Fig. 1 Construction and antigen specificity of chimeric IgE antibodies. **a** Graphical representations of the vector constructs. The parent vectors were pcDNA 3.1/neomycin (pEpsilon I), pEF6/blastacidin (pEpsilon II), pcDNA 3.1/Zeocin (pKappa I), and pcDNA 3.1/hygromycin (pKappa II). Heavy and light chain variable region genes were cloned from the hybridomas in Table 1 and inserted upstream of the human epsilon or human kappa constant region genes. **b** SDS-PAGE analysis. Chimeric IgE purified from transfected CHO-K1 clones and control human IgE from myeloma cell line SKO-007 were separated by electrophoresis on a 4–12 % gradient acrylamide gel and stained with Coomassie blue. **c** Analysis of antibody specificities. Histogram overlays depict FACS analysis of IgE binding to cell lines transfected with cognate antigen, compared to untransfected cell lines. 1F5.hIgE (anti-hCD20) bound A20 cells transfected with human CD20 cDNA, while 3C6.hIgE (anti-hMUC1, partially glycosylated form) detected human MUC1 on the mouse breast carcinoma cell line 4T-1 transfected with the hMUC1 cDNA

antigens are described in Supplementary Table 2. ELISAs were developed using tetramethylbenzidine (TMB) substrate (Dako Inc., Carpinteria, CA). The reaction was terminated with 2 M sulfuric acid, and plates were scanned at 450 nm using a SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

Antibody production in CHO cells

CHO clones were adapted to grow in 150-ml shaker flasks on an Innova 2000 platform shaker (New Brunswick Scientific, Edison, NJ) at 165 rpm, in a 37 °C humidified incubator with 5 % CO_2. Cells were adapted to serum free conditions with BD ACF media, supplemented with 1 % HEPES buffer, 1 % penicillin/streptavidin and 2 % pluronics. Adapted clones in log-phase growth were inoculated into 5-l Wave bioreactor™ bags (GE Healthcare, Waukesha, WI) at 0.5×10^6 cells/ml. Cells were then grown using the Wave bioreactor™ System2/10. Daily aliquots were taken, and the pH and cell concentrations measured. Oxygenation was determined using the dissolved oxygen monitor and DOOPT probe (GE Healthcare). CO_2 and O_2 input and mixing rates were adjusted as necessary. ACF media + supplements was added daily until the total volume reached 5 l. The culture was then transitioned to BD QY media by the daily addition of 1.25 l of QY media (+supplements) and harvest of 1.25 l of the total culture. After 20 l had been harvested, the process was terminated and the remaining media in the bag collected.

Media clarification and concentration of recombinant antibodies

CHO cell culture supernatants were clarified by pressure filtration through a Durapore 0.65-μm membrane filter (Millipore, Billerica, MA) on a 142-mm stainless steel filter holder (Millipore) and concentrated using a Labscale TFF system and Pellicon XL 50 100 K membrane (Millipore).

Next, ammonium sulfate (Sigma) was added to a concentration of 1.4 M. The mixture was stirred for 4 h, centrifuged at $10{,}000 \times g$ for 15 min, and the resulting supernatant harvested. More ammonium sulfate was then added to the supernatants to a concentration of 2.4 M. The mixture was stirred overnight at 4 °C, centrifuged, and the resulting pellet resuspended with 2 × pellet volume of PBS.

IgE column purification

To purify human IgE, omalizumab (Xolair®; Novartis Pharmaceuticals Ltd/Genentech, South San Francisco, CA) [9] was conjugated to CnBR-activated Sepharose beads (Invitrogen) at 10 mg/ml and packed into 6-ml glass columns. Columns were pre-washed with Antibody Gentle Binding Buffer (Pierce), before the sample was applied to the columns (0.75 ml/min). Columns were then washed to background, and the antibodies eluted with 0.2 M glycine (pH2.5), into 2 M Tris, pH8.0. The eluant was dialysed overnight into phosphate-buffered saline (PBS), concentrated using 50-kDa Vivaspin columns (Sartorius AG, Brentwood, NY), and quantitated using a BCA™ Protein Assay Kit (Pierce).

SDS-PAGE

1.5 μg of protein was boiled in SDS-PAGE buffer for 5 min, loaded on a NuPAGE 4–12 % Bis–Tris gel (Invitrogen), and separated by PAGE for 40 min at 200 V. A RPN800 V Rainbow marker (Amersham plc, Piscataway, NJ) was used to determine protein size. Proteins were visualized by staining with Biosafe Coomassie (BioRad Labs, Hercules, CA) for 1 h, and destaining in distilled water for 30 min prior to visualization.

Differentiation of mast cells and eosinophils from umbilical cord blood

Cultured human mast cells were derived essentially as described in Kempuraj et al. [10]. Briefly, mononuclear cells were isolated from heparinized umbilical cord blood. The cord blood was obtained from Lucille Packard Children's Hospital under a protocol approved by the Stanford University Institutional Review Board. Mononuclear cells were isolated using Ficoll-Paque™ Plus (GE Health Care Bio-Sciences, Piscataway, NJ). $CD133^+$ mononuclear cells were isolated using a magnetic separation column and an indirect $CD133^+$ cell isolation kit (Miltenyi Biotec, Auburn, CA). $CD133^+$ progenitor cells were maintained in culture medium consisting of Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.1 % bovine serum albumin (BSA; Sigma Aldrich, St Louis, MO); 80 ng/ml $rhSCF^{164}$; 50 ng/ml rhIL-6; and 1 % Insulin-Transferrin-Selenium, 10 mM HEPES, 2 mM L-glutamine, antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mg/ml gentamicin), 1 × MEM vitamin solution, 50 mM 2-ME, 1 × MEM amino acids and 1 mM sodium pyruvate. Recombinant hIL-3 (1 ng/ml) was added at the beginning of the culture; thereafter, half the volume of the culture medium (but without rhIL-3) was changed weekly. After 4 weeks, 10 % FBS was added. Cells from 11 to 14 weeks cultures were used for experiments, at which time mast cells accounted for >90 % of the total live cells as judged by their ability to bind antibodies to c-kit and human IgE (Supplementary Figure 1A).

Eosinophils were derived by culturing cord blood mononuclear cells at 1×10^6 cells/ml, in StemSpan® H3000 media, supplemented with 5 ng/ml rhIL3 and 5 ng/ml rhIL5. Half the volume of the culture medium was changed weekly. Cells from 3 to 6 weeks cultures were used for experiments, at which time eosinophils accounted for >95 % of the total live cells (Supplementary Figure 1B).

CBMC activation assay

Cord blood mast cells (CBMCs) were incubated overnight with 2 μg/ml of either SKO or 1F5.hIgE. The next day, cells were washed to remove excess IgE and plated into 96-well plates at 10^4 cells/well. Tumor cells were added to achieve different tumor-MC ratios, keeping the total volume at 100 μl/well. After 6 h, supernatants were harvested and hIL-8 concentration determined by ELISA.

Serotonin release assay

Peritoneal lavage was performed on 8–10-week-old hFcεRI mice, and the mast cells isolated by density centrifugation. Each mouse was lavaged with 10 ml lavage buffer (HBSS, 10 % FBS, 10 U/ml heparin). Lavage fluid was spun for 5 min at 1,000 rpm, and the cell pellet resuspended in 1.5 ml buffer and layered on top of a 23.5 % Histodenz (Sigma) solution. The gradient was spun at 1,350 rpm for 15 min, and the cell pellet recovered. Cell preparations were >90 % mast cells as determined by staining with toluidine blue.

Freshly isolated peritoneal mast cells were cultured overnight in 2 μg/ml 1F5.hIgE and 10 ng/ml stem cell factor (mSCF, Peprotech). Cells were washed once and then loaded with 1 μCi/ml 5-^{3}HT (Perkin Elmer) for 2 h at 37 °C. The cells were washed twice to remove excess radioactivity in the supernatants and put into 1.5-ml eppendorf tubes at 5×10^4 cells/tube. OCI-Ly8 cells or anti-IgE antibody was added, so the total volume was 100 μl. After 15 min, the tubes were spun, and supernatants harvested. The pellet was lysed in 100 μl 10 %NP-40 buffer. Radioactivity of both fractions was quantitated using a beta counter, and percentage serotonin release calculated using the formula:

$$\text{Percentage release} = \text{Activity in media}/(\text{activity in media} + \text{pellet}).$$

Flow cytometry (FACS)-based cytotoxicity assay

A flow cytometry assay was used to study tumor cell cytotoxicity and phagocytosis as previously described [7], with modifications. OCI-Ly8 human B cell lymphoma cells were washed twice in PBS and labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Sigma) for 15 min at room temperature. Labeled cells (1×10^5) were mixed with unstained effector cells at different effector-tumor ratios, but constant amounts of SKO or 1F5.hIgE (2.5 μg/ml (CBMCs) or 5 μg/ml (CBEos)). Reaction tubes were capped and incubated at 37 °C. For blocking experiments, 2 μg/ml blocking antibody or 10 U/ml heparin was added to the reaction mixture. All conditions were tested in triplicate. After 24 h, cells were stained with 0.25 μg/ml propidium iodide (Sigma) before analysis on a FACScan flow cytometer. Flow cytometry data were analyzed using FlowJo software.

Cytokine profiling of activated CBMCs

CBMCs were incubated overnight with 2 μg/ml SKO or 1F5.hIgE. Cells were washed to remove excess IgE and aliquoted into 96-well plates at 10^5 cells/well. Cells were then mixed with media alone, anti-IgE (1 μg/ml), or 2×10^5 OCI-Ly8 cells and incubated at 37 °C. After 24 h, supernatants were harvested and analyzed by ELISA or Luminex® (Luminex Corp, Austin, TX). Luminex® cytokine standards were analyzed by multiplex bead-based arrays using the Beadlyte Human 36-Plex Multi-Cytokine Detection System (Upstate-Cell Signaling Solutions, Lake Placid, NY) and analyzed on the Luminex® 200 instrument according to the manufacturer's protocol.

Mice and genotyping

Human FcεRIα-chain transgenic mice were a kind gift of J.P. Kinet (Harvard Institutes of Medicine, Boston, MA) and have been previously described [11]. At 3 weeks of age, mice were weaned and DNA obtained from digested toe clippings [12]. PCR genotyping was performed with the following primers: [hFcεRIα] GCTCCAGATGGCGTGTTAGC (5′) and TCCA GAAAGTAGTGAGAGGC (3′); [*neo*] TTGTTTTTCTCTT GATTCCCACTT (5′) and CTCATAATCTGTCCTTGGTC ACCTC (3′); [mFcεRIα] GGGATGGGTTTCAAGACTTAT TGGTTGGGTATTCCCTTAGCC (5′) and GAGATATG GACTCTCATAATGTGTCCTTGGTCACC (3′). BALB/c Ka mice were obtained from the breeding facility of the Department of Laboratory Animal Medicine at the Stanford University School of Medicine. The animal experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

In vivo IgE pharmacokinetics

Human IgE (100 μg) was injected into the tail vein (i.v.) or peritoneal cavity (i.p.) of hFcεRI transgenic mice, and anaesthesized mice were bled at 0, 1, 6, 12 and 24 h, retro-

orbitally using heparinized tubes. Serum was diluted 1:500, and hIgE quantitated by enzyme-linked immunosorbent assay (ELISA).

Subcutaneous tumor model

10^5 4T1 cells, in a total volume of 50 µl, were injected subcutaneously (s.c.) into the shaved right flank of each mouse. Either 1F5.hIgE or 3C6.hIgE (20 µg in 50 µl) was injected subcutaneously at the indicated time points. After approximately 5 days, the tumors became palpable, and their size was estimated by two-dimensional (2D) caliper measurements at various times.

Histology

Tumors were excised from mice and fixed in 10 % buffered formalin. Histology was performed by Histotec Laboratories (Hayward, CA). Paraffin-embedded sections were stained with hematoxylin and eosin, toluidine blue (mast cell detection) or the murine 3C6 mAb (anti-human MUC1).

Creation of 4T1 transfectants

The cDNAs encoding mouse IL-5 and MCP-1 were cloned and inserted into pcDNA3.1(+). Vectors encoding IL-5, MCP-1 and 3C6.mIgE were separately transfected into 4T1 cells using Fugene 6, to obtain the cell lines described in Table 2.

Statistical analysis

For analysis of the results of the multiplex cytokine assay, a 2-class significance analysis of microarrays (SAM) algorithm [13] was applied to the dataset. Cytokines were considered significantly upregulated in the activated CBMC group if they had a q value of <5 % and a fold change of >5.0 compared to the quiescent CBMC group. Heat map and Euclidean complete linkage hierarchical clustering images were generated using Tigr Multiexperiment Viewer (TM4:MeV; Dana-Farber Cancer Institute, Boston, MA) [14]. ANOVA, *t* tests, Kaplan–Meier survival curves, log-rank test and generation of graphs were performed using the Prism 4 software (GraphPad Software Inc, La Jolla, CA).

Results

Creation of chimeric IgE gene vectors

The two parent mouse antibodies (Abs) are described in Table 1. The 1F5 hybridoma targets human CD20 (hCD20), a pan-B cell marker and important therapeutic target for treatment of B cell lymphomas and several autoimmune diseases [15, 16]. VU-3C6 targets human mucin 1 (hMUC-1), a mucin overexpressed on tumors arising from glandular epithelium [17]. Antibody variable gene segments were cloned from each hybridoma and grafted onto human Ig kappa light chain and epsilon heavy chain genes. We designated the final chimeric antibodies 1F5.hIgE and 3C6.hIgE. The final plasmids are described in Fig. 1a.

Table 1 Hybridomas used to isolate variable gene segments

Hybridoma clone	Mouse isotype	Immunizing antigen	Chimeric IgE nomenclature
VU-3C6	IgG1	ZR75.1 human breast cancer cell line	3C6.hIgE
1F5	IgG2a	Human B cell lymphoma	1F5.hIg

Antibody production and purification

The final plasmids were transfected into CHO-K1 cells for antibody production. Stable transfectants were adapted to grow in shaker flasks and serum free media (BD Animal Component Free media, supplemented with 1 % HEPES buffer, 1 % penicillin/streptavidin and 2 % pluronics).

To further optimize antibody yields and scale up production, we utilized the Wave Bioreactor™ System2/10 (Wave) [18]. The Wave system enabled us to control temperature, aeration rate, pH and oxygenation, as well as feed and harvest media in a sterile fashion. Harvested CHO supernatant was filtered to remove cell debris and concentrated via tangential filtration. The protein components (Select Soytone) of the BD Animal Component Free media precipitated beyond 10 × concentration, clogging the filter membranes and leading to low yields. To overcome this, 10 × concentrated supernatants were subjected to a 2-step ammonium sulfate precipitation. The 2-step process involved one precipitation step at 1.4 M ammonium sulfate (IgE remains in solution, while Select Soytone precipitates), decanting the supernatant, and adding more ammonium sulfate to 2.4 M, precipitating the IgE. The 2.4 M pellet was resuspended in PBS, and human IgE was purified by Fast Protein Liquid Chromatography, using an anti-hIgE affinity column. Figure 1b shows the SDS-PAGE comparison of the purified chimeric IgEs, to a human IgE isotype control (IgE from human myeloma SKO, purified in a similar fashion). All three samples of chimeric antibody are pure, with minor size differences compared to control hIgE (likely due to different glycosylation patterns). Under reducing conditions, the epsilon heavy chain migrates at 75 kDa, unlike the gamma heavy chain, which migrates as a 50 kDa polypeptide.

Verification of antibody specificities

We tested the purified antibodies for their ability to bind their native antigens. As analyzed via flow cytometry, 1F5.hIgE bound the A20 mouse B cell lymphoma transfected with human CD20, but not the wild-type cell line (Fig. 1c, left panel). Similarly, 3C6.hIgE bound to 4T1 murine breast cancer cells transfected with human MUC1, but not to untransfected 4T1 cells (right panel). Thus, we conclude that the use of the human IgE constant regions, as well as our production and purification protocol, did not affect antigen recognition by chimeric antibodies.

Functional testing of 1F5.hIgE (anti-hCD20)

The 1F5.hIgE-CD20 system is easily manipulated in vitro and easily queried by flow cytometry, due to the ability to culture lymphoma cell lines A20 (mouse) and OCI-Ly8 (human) in suspension. Thus, we were able to rapidly investigate different mechanisms by which human mast cells and eosinophils may respond to IgE-coated tumor cells. Results of these investigations are presented in the following paragraphs. We did not test this system in vivo because a physiological model of lymphoma would result in significant levels of circulating antigen, leading to a high risk of rapid anaphylaxis upon treatment with IgE.

In vitro activation of mast cells by chimeric IgE and cell surface antigen

To determine whether the anti-hCD20 chimeric antibody 1F5.hIgE had a functional IgE Fc region, we investigated its ability to activate cord blood-derived mast cells (CBMCs). IgE-dependent activation of mast cells by the high affinity IgE receptor FcεRI is well described [19]. Mast cell activation by IgE results in the release of three classes of mediators: (a) preformed mediators stored in cytoplasmic granules, for example tryptase, TNF, histamine and serotonin (mouse only); de novo synthesis of (b) proinflammatory lipid mediators, for example prostaglandins; and (c) numerous cytokines, chemokines and growth factors, for example TNF, IL-8. Using interleukin-8 (IL-8) production as a measure of CBMC activation, we observed that CBMCs pre-coated with 1F5.hIgE produced IL-8 in the presence of hCD20-transfected mouse B cells (A20.hCD20), but not untransfected cells (Fig. 2a). Similarly, only CBMCs coated with anti-hCD20 IgE (1F5.hIgE) responded to the $hCD20^{+}$ human B cell OCI-Ly8, while CBMCs coated with a control IgE (SKO) failed to produce IL-8 under these conditions (Fig. 2b). These data demonstrate that 1F5.hIgE activates mast cells in an antigen-dependent and antigen-specific manner. We confirmed these results by assessing degranulation of peritoneal mast cells from hFcεRI transgenic mice upon stimulation with 1F5.hIgE and anti-hIgE or hCD20-expressing tumor cells (OCI-Ly8). Using serotonin release as a measure of degranulation, Fig. 2c shows that 1F5.hIgE triggered mast cell degranulation in an antigen-dependent manner.

Mast cells and IgE-mediated tumor cytoxicity

To test for potential cytotoxic effects mediated by tumor-specific IgE, we focused on effector cells known to express and respond to the high affinity IgE receptor, FcεRI. Previous investigators reported IgE-dependent tumor cytotoxicity mediated by human monocytes and eosinophils [7, 20]. Like Karagiannis et al., we observed IgE-dependent tumor cytotoxicity when we use the human monocyte cell line U937 as effector cells (data not shown). However, in this report, we focus on data obtained using human mast cells and eosinophils—two cell types involved in the pathogenesis and tissue damage observed in allergy and asthma [21–23]. Mast cells were of particular interest because they reside in the tissues where tumors arise, express high levels of FcεRI, and when activated trigger a coordinated inflammatory response that recruits eosinophils, neutrophils and other effectors that may mediate tumor regression [24]. Previously, mast cells have been shown to exert tumoricidal effects via TNF and the peroxidase system [25–27].

To test whether activated mast cells can directly induce tumor cell death, we prepared purified, cultured mast cells and incubated them with IgE and tumors. Cultured mast cells derived from cord blood (CBMCs) functionally resemble human mast cells that have been freshly isolated from tissues [28]. CBMCs were mixed with OCI-Ly8 B cells, in the presence of 2.5 μg/ml anti-CD20 (1F5.hIgE) or control (SKO) IgE. After 24 h, propidium iodide (PI) was added to label dead cells, and the mixture analyzed by flow cytometry. The percentage of $CFSE^{+}$ cells that were also PI^{+} indicated the fraction of dead/dying tumor cells present. An increase in tumor cytotoxicity was observed when 1F5.hIgE was added, compared to the control antibody (Fig. 3a). The magnitude of this effect was not augmented by increasing the effector:target ratio above 2:1. Using a previously published assay for antibody-dependent cell phagocytosis [7], mast cells were labeled with an antibody to c-kit and the percentage of c-kit+/CFSE+ cells measured in the presence of specific or control IgE. We did not observe any significant IgE-dependent phagocytic activity by mast cells (data not shown).

To investigate the mechanism by which CBMCs might induce tumor cell death, the cell mixtures were incubated with a series of blocking antibodies, or a rat IgG1 isotype control. The addition of anti-TNF decreased tumor cytotoxicity from 24.2 ± 3.5 % to 14.0 ± 0.3 % (Fig. 3b).

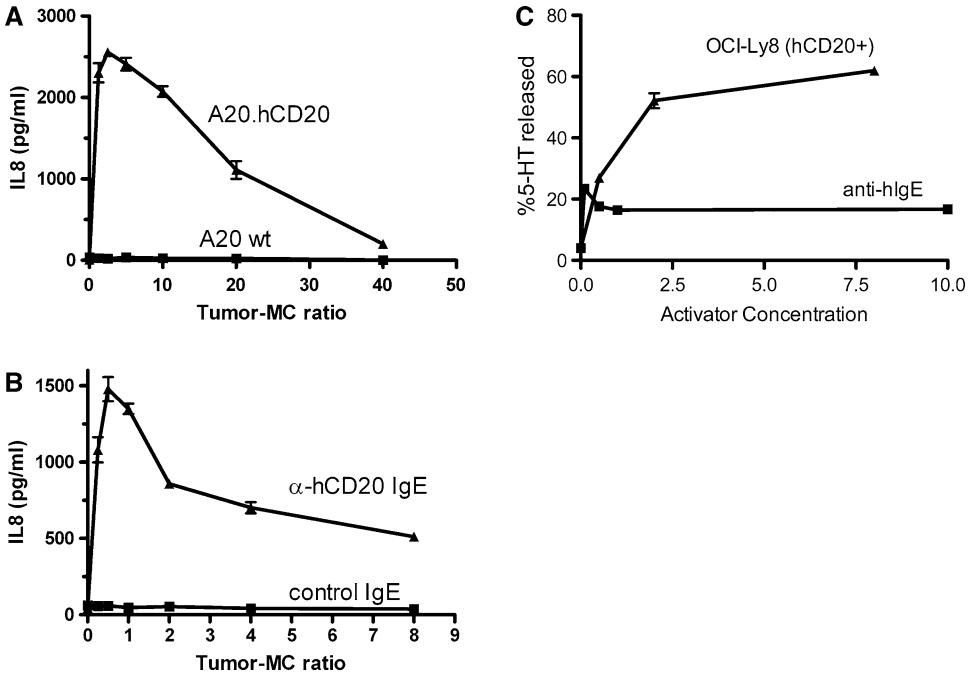

Fig. 2 Activation of mast cells by 1F5.hIgE and hCD20$^+$ tumor cells. *Graphs* show concentrations of IL8 released by activated cord blood-derived mast cells (CBMCs). **a** CBMCs were incubated overnight with 2 μg/ml 1F5.hIgE (anti-hCD20). The next day, cells were washed and plated into 96-well plates at 10^4 cells/well. A20 wild type or A20.hCD20 (hCD20$^+$) tumor cells were added at different tumor-MC ratios, keeping the total volume at 100 μl/well. **b** CBMCs were incubated overnight with 2 μg/ml SKO or 1F5.hIgE. The next day, cells were washed and plated into 96-well plates at 10^4 cells/well. OCI-Ly8 tumor cells (human B cell lymphoma, hCD20$^+$) were added at different tumor-MC ratios, keeping the total volume at 100 μl/well. **a** and **b** After 6 h at 37 °C, supernatants were harvested and IL8 concentration quantified via ELISA. *Error bars* represent mean ± standard deviation of duplicate wells. **c** Mast cells were isolated from abdominal lavage fluid of hFcεRI mice and cultured overnight with 1F5.hIgE. Cells were then loaded with radioactive serotonin (5-^{3}HT) and activated with either anti-hIgE (*square symbols*) or OCI-Ly8 tumor cells (*triangles*). After 15 min, the cells were centrifuged, and radioactivity in both the supernatant (*S*) and cell pellet (*P*) was quantitated. %5-HT release was calculated by % release = $S/(S + P)$. Activator concentration represents tumor-mast cell ratio (for OCI-Ly8) or μg/ml (anti-IgE). Results shown are Average ± SD from duplicate wells of one representative experiment

Slight reductions were seen with the other antibodies tested, but these differences were not statistically significant.

Eosinophils and IgE-mediated tumor cytotoxicity

Tumor eosinophilia has been associated with a favorable prognosis, especially in tumors of the gastrointestinal tract [29–31]. Previously, Karagiannis et al. have reported that eosinophils isolated from human peripheral blood mediate cytotoxicity when tested with the ovarian cancer cell line IGROV, in an IgE-dependent fashion [20]. To obtain sufficient numbers of naïve human eosinophils, we differentiated cord blood mononuclear cells in the presence of IL-3 and IL-5. Eosinophils obtained by this protocol exhibit phenotypic and functional similarities to peripheral blood eosinophils [32]. After 3 weeks, >95 % of live cells in cultures resemble mature eosinophils phenotypically (CD66b$^+$, CD16$^-$) as analyzed by flow cytometry (Supplementary Figure 1B) and visually confirmed by microscopy after staining with hematoxylin and eosin (data not shown). We designated these cells cord blood-derived eosinophils (CBEos). We mixed CBEos with OCI-Ly8 B cells and added 5.0 μg/ml of either control (SKO) or tumor-specific (1F5.hIgE) IgE antibodies. After 24 h at 37 °C, PI was added to label dead cells, and the mixture analyzed by flow cytometry (Fig. 3c). As observed with CBMCs, CBEos triggered increased tumor cell death in the presence of tumor-specific IgE compared to control IgE. Interestingly, the antibody dependence of this effect was less pronounced at higher effector-target ratios, suggesting that higher eosinophil to tumor ratios can lead to cell death in an antibody-independent fashion.

To investigate the mechanism of CBEos-mediated tumor cytotoxicity, we incubated the cultures with a panel of blocking antibodies and inhibitors (Fig. 3d). We observed a modest decrease in tumor death with blocking antibodies to TNF-related apoptosis inducing ligand (TRAIL), and a more significant effect upon addition of a low concentration of heparin (10 U/ml). Heparin, an anionic molecule, is thought to exert this effect by neutralizing the cationic proteins released by eosinophils (eosinophil cationic protein (ECP), major basic protein

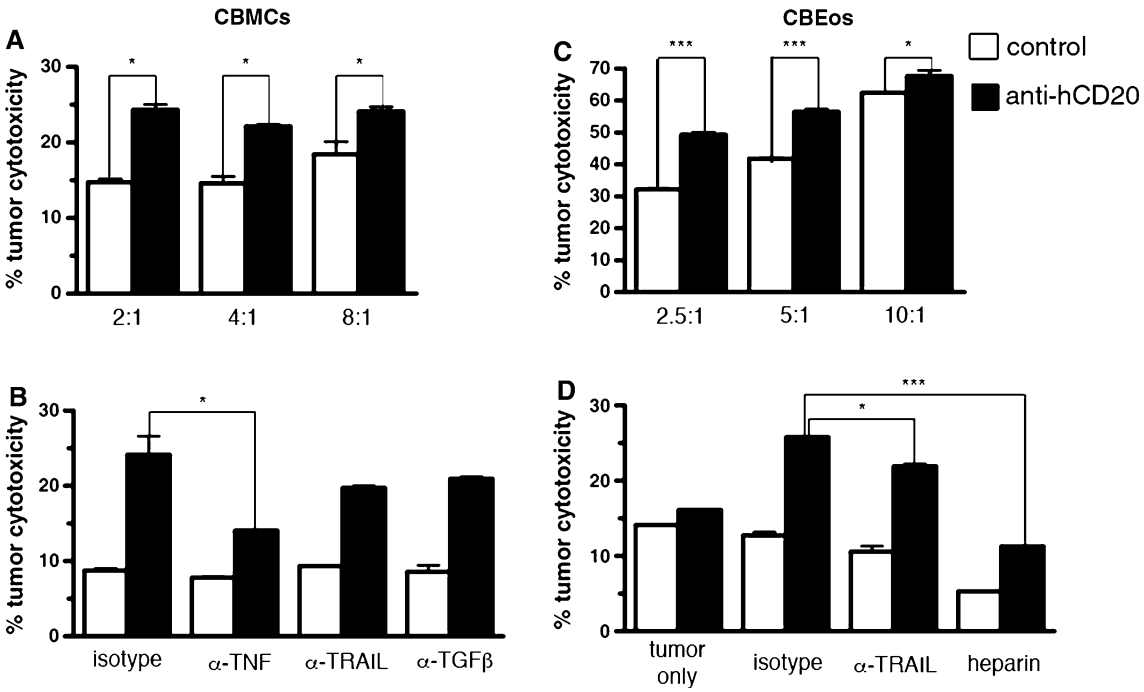

Fig. 3 Mast cell and eosinophil-mediated tumor cytotoxicity in vitro. Histograms show % PI^+CFSE^+ tumor cells. OCI-Ly8 human B cell lymphoma cells ($hCD20^+$) were labeled with 10^{-5} mM CFSE for 15 min. 1×10^5 labeled cells were mixed with unstained CBMCs and 2.5 μg/ml IgE (**a** and **b**) or cord blood-derived eosinophils (CBEos) and 5 μg/ml IgE (**c** and **d**), then incubated at 37 °C for 24 h. The cell mixture was stained with propidium iodide (PI) before flow cytometric analysis. The percentage of PI^+ cells in the $CFSE^{hi}$ fraction represents total tumor cytotoxicity, and results are shown in histogram form. **a** CBMC- and hIgE-mediated tumor cytotoxity at different effector:target ratios. **b** 2μg/ml blocking antibodies or isotype control were added (E:T ratio 4:1). **c** CBEos- and hIgE-mediated tumor cytotoxicity at different effector:target ratios. **d** 2 μg/ml blocking antibodies or 10 U/ml heparin was added. (E:T ratio 2.5:1). Also shown here are data for tumor death induced by antibodies alone (i.e., in the absence of effector cells). Results shown are mean ± SD of one representative experiment. Student's t test *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$

(MBP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN)) [33]. These cationic proteins have been shown to cause eukaryotic cell death by disrupting negatively charged cell membranes [34].

A higher IgE concentration was used for CBEos (5.0 μg/ml) compared to CBMC (2.5 μg/ml), due to the reduced magnitude of the CBEos response at the lower IgE concentration. Although the trends were similar at both concentrations, the reduction in cytotoxicity upon addition of anti-TRAIL and heparin was not statistically significant at 2.5 μg/ml IgE, when $n = 4$ for each group. The requirement for different IgE concentrations may be due to differences in surface expression of FcεRI between the two cell types.

Cytokine profiling of activated CBMCs

To predict the effectiveness of tumor-specific IgE in vivo, we investigated whether IgE-coated tumor cells were able to trigger the full spectrum of mast cell cytokine production. A large number of genes are upregulated by activated mast cells upon their activation by IgE and antigen [35]. To investigate which cytokines/chemokines are produced by mast cells activated by tumor-specific IgE, we performed an unbiased screen for cytokines. Supernatants from mast cells activated by coculture with IgE and tumor cells were analyzed for 36 cytokines using a multiplexed bead-based assay (Fig. 4). CBMCs were activated through FcεRI for 24 h at 37 °C by two methods: coculture with IgE and anti-IgE, or by coculture of anti-hCD20 IgE with hCD20-expressing tumor cells. A panel of inflammatory, growth and chemotactic factors were assessed. To identify cytokines significantly upregulated in activated versus resting mast cells, we applied a 2-class SAM algorithm ($q < 0.05$, fold change > 5.0) (Fig. 4b). SAM identified inflammatory cytokines such as macrophage inflammatory protein (MIP)-1α, MIP-1β, granulocyte macrophage colony-stimulating factor (GM-CSF), epithelial neutrophil activating peptide 78 (ENA78) and IL-8. As expected, the strength of the response was greater when mast cells were activated with a multi-valent antigen (e.g., a cell surface antigen), than when activated by simple bivalent crosslinking (IgE + anti-IgE). Hence, we predict that mast cells encountering IgE-coated tumor cells in vivo should trigger the classic IgE-induced inflammatory response. However, we could not account for immune-modulatory signals that may be present in the tumor microenvironment.

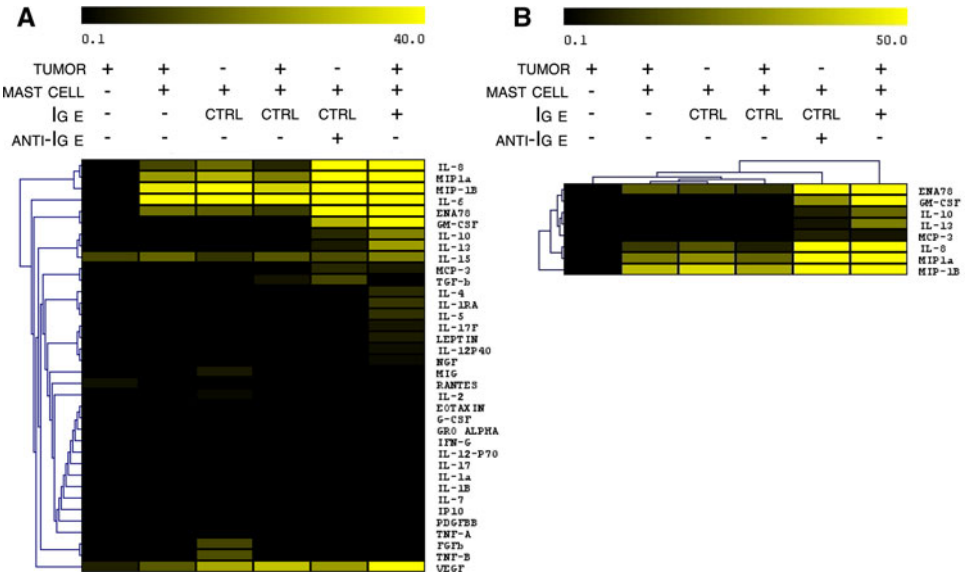

Fig. 4 Cytokine profiling of activated cord blood-derived mast cells. CBMCs were incubated overnight in the presence or absence of 2 μg/ml hIgE, and 10^5 cells were mixed with 1 μg/ml anti-IgE or 2×10^5 OCI-Ly8 lymphoma as indicated, in a total volume of 100 ul. After 24 h, supernatants were harvested and analyzed via a Luminex® 36-plex human cytokine assay. Cytokine concentrations were determined from a standard curve generated with Luminex® standards and bead pairs. **a** Hierarchical clustering of cytokine concentration values for all 36 cytokines. *Columns* correspond to supernatants from cells treated under different conditions. *Column 1*: tumor only; *Columns 2*, *3*, *4*: mast cells ± control IgE ± tumor; *Columns 5* and *6*: mast cells activated via IgE + anti-IgE and IgE + cell surface antigen, respectively. The intensity of the *yellow bars* represents increasing cytokine concentration. **b** Heat map displaying hierarchical clustering of cytokines identified by significance analysis of microarrays (SAM) as significantly upregulated (q value < 0.05, fold change >5.0) in activated (*Columns 5* and *6*) versus quiescent (*Columns 2–4*) mast cells

Functional testing of 3C6.hIgE (anti-hMUC1)

Although testing of 1F5.hIgE demonstrated promising results in vitro, we were more intrigued by the possibility of using IgE against antigens expressed on solid tumors, which are less tractable to conventional IgG therapy [36]. Therefore, we constructed 3C6.hIgE against the human MUC1 antigen, which is overexpressed on tumors arising from glandular epithelium, such as the breast, ovary and colon [17]. The 3C6.hIgE-MUC1 system is not easily amenable to flow cytometry, due to the adherent nature of epithelial cell lines. Therefore, we went directly to an in vivo model of transplantable tumor, based on the well-described murine breast carcinoma line 4T1 [37].

Creation of 4T1.hMUC1 cell line

To test the in vivo tumoricidal capacity of anti-hMUC1 IgE (3C6.hIgE), we created a murine cell line that expressed the transmembrane form of hMUC1. We transfected the full-length hMUC1 cDNA into the murine breast carcinoma 4T1 and named the transfected line 4T1.hMUC1. 4T1 was isolated from a spontaneously arising mammary carcinoma in a Balb/c mouse [37] and has been used as a transplantable model of breast cancer [38]. 4T1.hMUC1 expresses abundant hMUC1 on its cell surface (data not shown) and grows subcutaneously in hFcεRI transgenic mice with kinetics similar to those observed for the parental 4T1 cell line (Supplementary Figure 2A).

Human FcεRI mouse model

To study the in vivo effects of targeting tumors with chimeric human IgE antibodies, we used a human FcεRIα transgenic mouse (hFcεRI Tg^+). In these mice, the endogenous gene encoding the α-subunit of the high affinity IgE receptor, FcεRIα, has been disrupted, and the mice are transgenic for the human homolog, under the control of the human FcεRIα promotor [11]. In contrast to wild-type mice, where FcεRIα expression is limited to mast cells and basophils, the range of expression of FcεRIα in hFcεRI Tg^+ mice resembles that seen in humans. In addition to mast cells and basophils, in hFcεRI Tg^+ mice, FcεRI is expressed on monocytes, Langerhans cells, B cells and eosinophils [39, 40]. The hFcεRIα gene product has the capacity to complex with the mouse beta and gamma subunits to form a functional 4 chain receptor ($\alpha\beta\gamma_2$). hFcεRI Tg^+ mice mount an anaphylactic response to human IgE antibodies and allergen [11].

To verify the ability of these mice to respond to human IgE, we administered 4T1.hMUC1 tumor cells into the peritoneum, followed by either control (SKO) or anti-

hMUC1 (3C6.hIgE) human IgE on day 9. After 24 h, peritoneal lavage was performed, the cells collected, cytospins made, and stained with hematoxylin, eosin and toluidine blue. Mast cells from the control group were intact (Supplementary Figure 2B), while those from the anti-hMUC1 group showed clear evidence of degranulation (Supplementary Figure 2C). This indicates that mast cells from hFcεRI Tg^+ mice are able to respond to human IgE in an antigen-specific manner.

Tumor-specific IgE inhibits in vivo tumor growth

The capacity of hMUC1-specific IgE to affect 4T1.hMUC1 tumor growth in vivo was tested in hFcεRI Tg^+ mice. For these experiments, we considered the intravenous and intraperitoneal delivery of IgE. We found that IgE is rapidly cleared in vivo (Supplementary Figure 3). This observation along with the fact that subcutaneous tumors are not well vascularized led us to administer the drug in the peritumoral region.

4T1.hMUC1 tumor cells (a total of 10^5) were inoculated subcutaneously (s.c.) into the shaved flanks of mice and treated with 20 μg SKO or 3C6.hIgE on days 1, 2, 3, 4 and 5 (Fig. 5a). We observed a modest inhibition of tumor growth in mice treated with 3C6.hIgE (24 % reduction in tumor size, $p < 0.001$ by two-way ANOVA). Also, only 3 of 8 mice in the control group survived to day 34, while 5 of 8 mice in the anti-hMUC1 group were still alive.

Tumor samples were obtained from surviving mice at day 34 and stained for the presence of mast cells using toluidine blue (Fig. 5c). We detected the presence of a few isolated mast cells in tumors from mice treated with control IgE. These cells were mostly peri-tumoral and did not demonstrate significant degranulation. We observed greater numbers of mast cells adjacent to the tumors in 3C6.hIgE-treated mice. These cells were in regions of necrosis surrounding the main tumor mass and demonstrated central clearing of toluidine blue-stained granules, indicating degranulation. Tumors retained hMUC1 expression even after treatment with anti-hMUC1 IgE (3C6.hIgE), as analyzed by immunohistochemistry (Supplementary Figure 4).

Enhanced tumor responses with local delivery of tumor-specific IgE and chemokines

The absence of clinically significant anti-tumor responses in our in vivo model may be due to two factors: first, the inability to deliver adequate amounts of antibody to a poorly vascularized and rapidly growing subcutaneous tumor; and second, the lack of sufficient effector cells in the microenvironment of these transplanted tumors. To test these variables, we created 4T1 cell lines that produced anti-hMUC1 mouse IgE (mIgE) or chemoattractant cytokines MCP-1 and

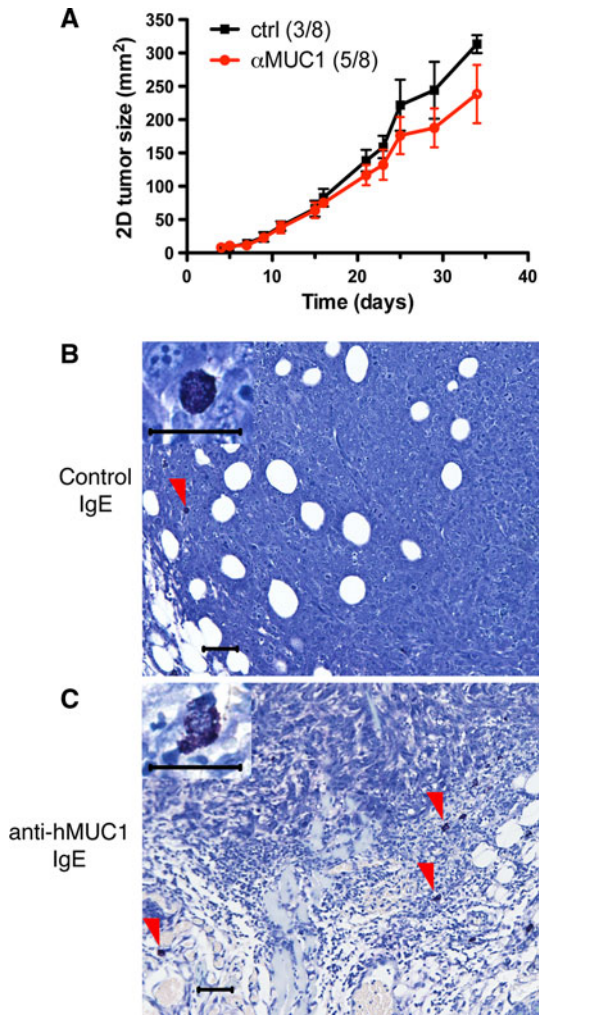

Fig. 5 Tumor-specific IgE inhibits tumor growth in vivo. 10^5 4T1.hMUC1 tumor cells were inoculated s.c. into the flanks of hFcεRI mice at d0. 20 μg control or 3C6.hIgE (anti-hMUC1) was administered at d1, 2, 3, 4, and 5. Two-dimensional caliper measurements were taken until tumors exceeded 300 mm^2 in area. **a** Graph of average tumor size against time. *Error bars* represent mean ± SD of each group. The number of surviving/total mice per group at the last time point is indicated in *brackets*. The anti-hMUC1 group is significantly different from the control group (two-way ANOVA, $p < 0.001$). **b** and **c** Tumors were harvested from surviving mice at d34 from the experiment shown in (**a**), sectioned, and stained for mast cells (*red arrows*). **b** In mice treated in control IgE, a few mast cells were present in peri-tumor regions, but they were mostly not degranulated. **c** In mice treated with 3C6.hIgE, more mast cells were present in necrotic peri-tumor regions. These cells exhibited central clearing of toluidine stained granules. Representative sections taken at ×10 magnification (scale bar = 20 μm), insets taken at ×60 magnification (scale bar = 50 μm)

IL5 (Table 2). We chose MCP-1/CCL2 because of recent reports that this cytokine is produced by tumor cells in response to oncogene activation and may be responsible for chemotaxis of monocytes and mast cells into the peritumoral stroma [41]. Interleukin 5 is both a growth factor and chemotactic factor for eosinophils [42]. We mixed combinations

Table 2 4T1 derivative cell lines

	4T1/3C6.mIgE	4T1.hMUC1/IL-5	4T1.hMUC1/MCP-1
Cell surface hMUC1 expression	–	+	+
Anti-hMUC1 mouse IgE production	1 ug/ml[a]	–	–
Mouse IL-5 production	–	10 ng/ml[b]	–
Mouse MCP-1 production	–	–	1 ng/ml[b]

[a] Measured in a mouse IgE-specific ELISA assay

[b] Measured at confluence in a mouse cytokine-specific capture ELISA assay

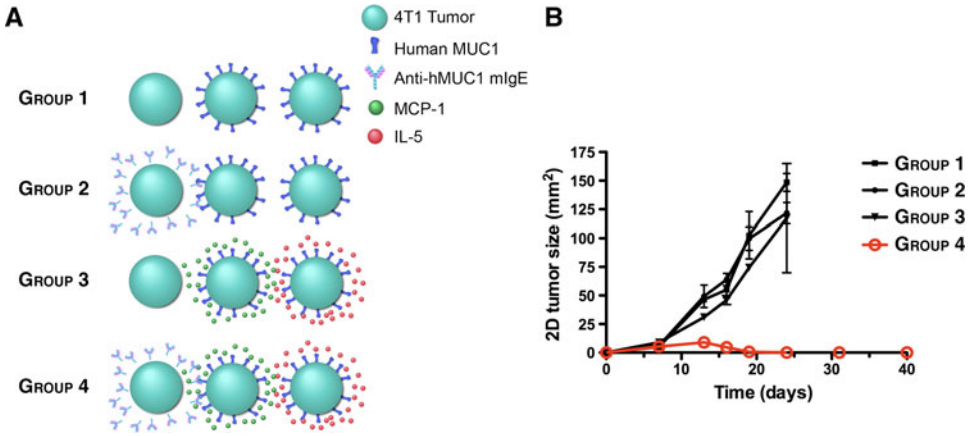

Fig. 6 Local production of IgE and chemokines prevents tumor growth in vivo. **a** and **b** Four different combinations of 4T1 tumor cells transfected with human MUC1, anti-hMUC1 IgE (3C6.mIgE) and/or cytokines (MCP-1, IL-5) were inoculated s.c. into the flanks of hFcεRI Tg^+ mice at d0. 10^5 total cells were administered per mouse ($n = 4$ per group). **a** Description of the four experimental groups. **b** Graphs of average tumor size against time. Two-dimensional caliper measurements were taken as described in "Materials and methods" and in Fig. 5. Data shown are representative of two separate experiments. *Error bars* represent average tumor size ± SD

of the antigen/cytokine producing 4T1 cells with mIgE producing 4T1 cells and injected them subcutaneously in hFcεRI Tg^+ mice (Fig. 6). Mixtures of antibody producing and antigen expressing tumors grew progressively as did tumors that expressed both MCP-1 and IL-5 but lacked local antibody production. In contrast, tumors that expressed anti-hMUC1 mouse IgE, the target antigen, and both cytokines failed to grow in 7 of 8 mice. In the single mouse that developed a tumor, it was only visible beginning on day 19 instead of day 8. Eosinophil-containing immune infiltrates were observed in tumors expressing MCP-1 and IL5 (data not shown). However, tumor elimination was only observed when tumor-specific IgE was also present. These data suggest that when sufficient amounts of tumor-specific IgE are delivered to an antigen-bearing tumor, in the presence of allergic effector cells, a complete and durable response can be observed.

Interestingly, when wild-type 4T1 tumor cells (producing neither hIgE nor chemokines) were injected s.c. into the opposite flank of the 7 mice which had rejected their tumors, the wild-type tumors failed to develop after 30 days (data not shown). These data suggest that IgE + chemokine-transfected tumor cells may be used prophylactically as a tumor vaccine.

Discussion

The mammalian immune system is a complex interplay of different specialized cell types from both the innate and adaptive immune arms. While there has been extensive interest and efforts in enhancing both effector and memory T cell responses to tumor antigens, mechanisms triggered by tumor-specific IgG antibodies have had the most clinical success to date.

The branch of the immune system activated by IgE antibodies (here termed the 'allergic immune system') is very powerful. Chronic activation of mast cells and eosinophils in the lung of allergic asthma patients produces airway remodeling and epithelial cell damage [43]. The cytotoxic effects of eosinophils against *Schistosoma mansoni* larvae, an organism many times the size of eukaryotic cells, are well documented [44]. Despite their known toxicities, the use of tumor-specific epsilon antibodies as cancer therapeutics has not been extensively studied. Our hypothesis was that the allergic immune system, triggered by tumor-specific IgE, may engage effector cells and demonstrate tumor responses in settings not seen with IgG1 mAbs.

A landmark study by Bruggemann et al. [5] compared the capacity of different antibody isotypes to kill tumor

cells via complement lysis and antibody-dependent cell-mediated cytotoxicity (ADCC). The IgG1 isotype was highly effective in both assays. This and other studies led to the use of gamma 1 as the predominant isotype used for therapeutic monoclonal antibodies. IgE antibodies do not fix complement and did not induce tumor death in that particular ADCC assay. However, the effector cells used were peripheral blood mononuclear cells (PBMCs), which contain extremely low proportions of the cells best able to be triggered by surface-bound IgE antibodies: mast cells, basophils and eosinophils. In this paper, we developed an assay utilizing mast cells and eosinophils as effectors and showed that IgE antibodies are indeed capable of carrying out ADCC. IgE crosslinking by cell surface antigen on tumor cells was sufficient to trigger effector cell activation, and this activation program induced target cell death under the conditions tested. Although our effector cells used here were derived from umbilical cord progenitors, they have been found to resemble, both phenotypically and functionally, effector cells isolated by conventional methods [32, 45].

A more rigorous test of the utility of these antibodies is whether they can alter tumor growth in vivo. Murine studies with IgE antibodies are complicated by two factors. First, human IgE does not bind the high and low affinity mouse IgE receptors, FcεRI and CD23, respectively, so human IgE cannot be directly tested in wild-type mice. Second, the mouse high affinity IgE receptor has a more restricted cellular expression pattern as compared to its human counterpart. To overcome these issues, we employed a mouse model first used in the allergy and asthma field. The hFcεRI transgene in these mice has a range of expression that resembles that found in humans. The hFcεRIα protein product can also bind and respond to both human and mouse IgE [11].

Using this transgenic mouse, we found that anti-hMUC1 IgE antibodies alone induced a modest inhibition of 4T1.hMUC1 tumor growth in vivo, as compared to control IgE. However, the 4T1 tumor is highly avascular and grows as a densely packed tumor mass, which may impede drug delivery and rapid effector cell recruitment. To overcome these limitations, we engineered 4T1 lines that express anti-hMUC1 mouse IgE, as well as the chemokines IL-5 and MCP-1. The presence of all three factors resulted in tumor eradication for greater than 30 days.

Our in vivo data emphasize the importance of antibody delivery to the tumor and the presence of effector cells in the tumor microenvironment. Even with early treatment and peritumoral injections of anti-MUC1 hIgE, significant, but modest, tumor responses were observed. This observation highlights the limitation of the transplantable tumor model when studying antibody-based therapies. Penetration of a large protein (~170 kD) into a small, unvascularized tumor likely constitutes a major limiting step in measuring effects on tumor growth. It is possible that more pronounced tumor effects could be seen with higher doses and more frequent antibody administration systemically. We are unable to intensively treat these immunocompetent mice with human IgE for greater that 10–14 days without the formation of mouse anti-human (MAHA) immunoglobulin responses (data not shown). Also, since 4T1 has a short latency period and grows rapidly, migration of allergic effectors under the influence of tumor derived factors may not occur to a sufficient degree to see a response even if the antibodies were abundantly bound to the tumor cell surface. Lastly, since 4T1 is tumor cell line, maintained in vitro, it may have lost expression of many factors that attract myeloid cells to the tumor stroma, as has been described for genetically driven tumors [41, 46, 47].

To address these deficiencies in the mouse model, we engineered 4T1 to express the heavy and light chains of mouse IgE-specific for human MUC1. This insured that the tumor was continually exposed to the antibody. Second, because the heavy and light chains were fully mouse, it obviated the possibility of a MAHA response from the mouse. Third, expression of two cytokines from the hMUC1 expressing 4T1 cells increased the frequency of two key effector cells that were likely to be important in this model, based on previous observations. These cytokines reconstitute the key effector cells seen predominantly in IL-4-transfected tumors [48, 49]. Consistent with previously published results with IL-5, simply expressing chemotactic cytokines in tumors (other than IL-4) failed to alter tumor growth kinetics [50]. In contrast, activation of these myeloid-derived cells, in this case by IgE and tumor antigen, allowed them to carry out their cytotoxic program leading to lack of tumor growth.

Although it is difficult to compare the two systems, it would appear that the in vitro assays underestimated how well the in vivo tumor suppression would work. Our in vitro data were optimized to highlight IgE-dependent tumor killing, not IgE-independent mechanisms. We observe, at high effector to target ratios, antibody-independent killing mechanisms of both CBMC and CBEos. The observation that 4T1/3C6.mIgE (hMUC1 negative) failed to emerge after eradication of 4T1.hMUC1/IL5 and 4T1.hMUC1/MCP1 suggests either a collateral damage/bystander effect or epitope spreading, or both. This observation is reminiscent of mixed tumor transplantation experiments showing elimination of wild-type tumors when mixed with IL-4 expressing tumors [48]. We speculate that early activation and amplification of the response is antigen and antibody dependent. As the response amplifies and more effector cells are recruited to the tumor, the response may take on an antibody-independent dimension, yet continue to be localized and controlled.

Remarkably, mice that rejected these tumor-specific IgE and chemokine expressing transplants also rejected subsequent injections of wild-type 4T1 cells (hMUC-1 negative), 30 days later. This not only suggests that epitope spreading had occurred, but the development of memory responses as well.

In conclusion, we report that mouse–human chimeric IgE antibodies specific for cell surface tumor antigens can activate both mast cells and eosinophils in vitro to carry out a cytotoxic program. This program is likely complex and at a minimum involves cytokines and released cationic proteins from the eosinophils. In vivo, the chimeric IgE antibodies have modest effects when administered to avascular subcutaneous tumors, but suppressed tumor growth and likely mediated complete tumor elimination when delivered with the tumor in the presence of cytokines chemotactic to cells of the allergic immune system.

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Conflict of interest The authors declare that they have no conflict of interest.

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