

ORIGINAL ARTICLE

Harnessing engineered antibodies of the IgE class to combat malignancy: initial assessment of FcεRI-mediated basophil activation by a tumour-specific IgE antibody to evaluate the risk of type I hypersensitivity

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Summary

Background IgE antibodies, sequestered into tissues and retained locally by the high-affinity IgE receptor, FcεRI, on powerful effector cells such as mast cells, macrophages and eosinophils, may offer improvements in the therapy of solid tumours. The chimeric antibody, MOv18 IgE, against the human ovarian carcinoma antigen, folate receptor α (FRα), is more effective than its IgG1 counterpart in xenograft models of ovarian cancer. Although MOv18 IgE binds to a single epitope on FRα and cannot cross-link IgE receptors on basophils, there remains a risk that components in the circulation of ovarian cancer patients might cross-link FRα-MOv18-IgE-receptor-FcεRI complexes on basophils to cause type I hypersensitivity.

Objective To assess the propensity for MOv18 used in a therapeutic setting to cause FcεRI-mediated type I hypersensitivity.

Methods As validated readouts of the potential for MOv18 to cause FcεRI-mediated type I hypersensitivity we measured release of a granule-stored mediator from a rat basophilic leukaemia cell line RBL SX-38 stably transfected with human tetrameric (αβγ2) FcεRI, and induction of CD63 on blood basophils from patients with ovarian carcinoma and healthy controls *ex vivo*.

Results Serum FRα levels were increased in ovarian cancer patients compared with healthy controls. MOv18 IgE alone, or in the presence of its antigen recombinant human FRα, or of healthy volunteer (*n* = 14) or ovarian carcinoma patient (*n* = 32) sera, did not induce RBL SX-38 cell degranulation. Exposure to FRα-expressing ovarian tumour cells at target-to-effector ratios expected within tumours induced degranulation. MOv18 IgE did not induce expression of CD63 in blood basophils from either healthy volunteers (*n* = 6), or cancer patients, despite detectable levels of circulating FRα (*n* = 5).

Conclusion and Clinical Relevance These encouraging data are compatible with the hypothesis that, when ovarian carcinoma patients are treated with MOv18, FcεRI-mediated activation of effector cells occurs within the tumour mass but not in the circulation mandating, with due caution, further pre-clinical studies.

Keywords anaphylaxis, basophil, cancer therapy, CD63, degranulation, FRα, tumour antigen-specific IgE, type I hypersensitivity

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Introduction

Monoclonal antibodies have become an important treatment modality in the clinical management of both haematological and solid malignancies in the last 25 years [1]. Nevertheless, many solid tumours such as those of the ovary remain refractory to treatment, indicating a need to improve antibody therapeutics, with a particular emphasis on localization of the antibody and initiation of anti-tumour immunity within the tumour mass itself.

We have previously investigated means of improving antibody therapies for solid tumours, using antibodies of the IgE class [2–7]. Exploitation of the superior tissue bioavailability and higher Fc receptor affinity of IgE, compared with IgG antibodies may translate to improved anti-tumour efficacy. IgE-mediated immune responses play a role in allergic inflammation and in protection against parasitic infections [8, 9]. Previous studies have demonstrated that IgE-mediated immune responses may be harnessed against malignant cells in tissues [7, 10–14].

MOv18 IgE is a chimeric antibody directed against the tumour-associated antigen folate receptor α (FR α). We have shown that it has superior efficacy against solid ovarian tumours compared with its IgG1 counterpart *in vitro* and in two *in vivo* cancer models [2, 7]. This antibody may represent a novel paradigm for the treatment of solid malignancies. FR α , a glycosylphosphatidylinositol-anchored membrane protein that binds folic acid, is an emerging therapeutic target for ovarian cancers [15–17]. It is expressed in 90% of epithelial ovarian carcinomas and in other types of cancer, but has limited expression in normal tissues [18, 19]. Elevated FR α expression on ovarian cancer tumour cells correlates with more aggressive disease and a worse prognosis [18]. Thus FR α would appear to be a prime molecular target for ovarian carcinoma and this, together with the aforementioned data supporting the therapeutic efficacy of MOv18 IgE pre-clinically supports our enthusiasm for development of this antibody for clinical studies [2–5, 7].

IgE antibodies can exert anti-tumoural effects by activating potent Fc ϵ R-expressing effector cells within tumours. We have previously reported evidence suggesting that MOv18 IgE can direct monocytes and eosinophils to kill tumour cells by mechanisms such as antibody-mediated cytotoxicity and/or phagocytosis [2–4]. In general, however, the interactions between this IgE and potent IgE receptor-expressing immune effector cells, such as basophils (in the circulation and potentially within tumours) and mast cells (focused in tumour microenvironments), have not been extensively examined [5, 20–22].

To date, IgE has never been used as a therapeutic agent in patients, and the administration of IgE directed against a tumour antigen raises the theoretical possibility of induction of type I hypersensitivity through cross-linking of IgE molecules bound to Fc ϵ RI on mast cells and

basophils. It is a requirement, however, that antigens capable of doing this have two or more IgE binding epitopes [23, 24] whereas MOv18 binds to only one single epitope on FR α [25, 26]. Consequently FR α , at least free and in solution, would not be expected to have the potential to cross-link surface-bound MOv18 bound to Fc ϵ RI although this could at least theoretically occur in the tumour cells expressing multiple surface FR α molecules within tumour islands.

In this study, we set out to investigate the capacity of FR α both in solution and expressed on ovarian carcinoma cells to activate basophils through MOv18 bound to human Fc ϵ RI. We adopted two assays used in the allergy field to examine sensitivity to a range of potential allergens in the presence of sera and blood of allergic individuals. We measured effector cell activation by functional degranulation with the humanized rat basophilic leukaemia (RBL) SX-38 cell line, and used the basophil activation test (BAT), based on detection of the activation marker, CD63, on basophils in the blood of healthy volunteers and ovarian carcinoma patients. With these experiments we account for any factors in blood that might recognize the tri-molecular complexes of FR α -MOv18 IgE-Fc ϵ RI on basophils, which could contribute to a risk of type I hypersensitivity in the proposed immunotherapy.

Methods

Human subjects and sera

Following written informed consent, sera from the peripheral blood of patients with stage III and IV ovarian cancer or healthy volunteers were collected and isolated by centrifugation of clotted blood at 1800 g for 15 min at room temperature. Sera were also collected from ovarian cancer patient peripheral blood as part of baseline studies before a clinical trial [27]. For the basophil activation assay (BAT), whole blood specimens of patients with stage III and IV ovarian cancer or healthy volunteers were freshly collected in 1.8 mg/mL K₂EDTA and assayed immediately.

Cell culture

Cells of the RBL SX-38 RBL cell line stably expressing the human tetrameric ($\alpha\beta\gamma_2$) high-affinity IgE receptor, Fc ϵ RI [28], (a kind gift from Prof. J-P. Kinet, Harvard University, Boston, MA) were cultured in EMEM, 10% FCS, 1.2 mg/mL Geneticin G418 (Gibco, Invitrogen, Paisley, UK). Human ovarian carcinoma IGROV-1 cells [3, 7], naturally over-expressing the human FR α , were cultured in RPMI 1640, 10% FCS and murine colon adenocarcinoma cells CT26 [6] in IMDM, 5% FCS. Cultures were maintained in a humidified incubator at 37 °C.

Flow cytometric assessments of MOv18 immunoglobulin E and antigen binding to effector cells

RBL SX-38 cells (2×10^5 cells per sample) were incubated with 0.5 $\mu\text{g/mL}$ MOv18 IgE in 0.2 mL FACS buffer (PBS, 5% FCS, Invitrogen), followed by 3 $\mu\text{g/mL}$ goat anti-human IgE-FITC (Vector Labs, Peterborough, UK) or 200 ng/mL human recombinant FR α [25], 3 $\mu\text{g/mL}$ goat anti-human FR α (R&D Systems, Abingdon, UK) and donkey anti-goat-Ig-Alexa Fluor[®] 448, (all in FACS buffer). For MOv18 IgE binding to human basophils in unfractionated blood, 100 μL blood diluted 1 : 1 with FACS buffer was incubated with MOv18 IgE, anti-IgE-FITC and mouse anti-CD123-RPE-Cy5 (BD Biosciences, Oxford, UK) or isotypic control IgG1/RPE-Cy5 (1 $\mu\text{g/mL}$, Dako, Stockport, UK) for 30 min at 4 °C. Red blood cells were removed with 2 mL lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 100 μM EDTA) for 10 min at room temperature. Cells were acquired on a dual laser FACSCanto (BD Biosciences) until at least 500 basophil (SSC^{low} CD123^{high}) events were recorded per sample.

Rat basophilic leukaemia SX-38 degranulation assays

We assessed degranulation of RBL SX-38 cells by measuring β -hexosaminidase release, as before [6, 28, 29]. Controls were: unstimulated cells; Triton X-100 lysed cells (to quantify 100% granule release); chimeric NIP IgE against the hapten 5-iodo-4-hydroxy-3-nitrophenyl (AbD Serotec, Kidlington, UK) with or without polyclonal antigen NIP-bovine serum albumin (BSA) [30]; NIP IgE plus polyclonal rabbit anti-IgE (Dako). Cells were seeded at 1×10^4 cells per well in culture medium overnight and sensitized with IgE (200 ng/mL), control antibody or medium alone. Samples were incubated at 37 °C for 1 h, washed 3 $\times$ in stimulation buffer (HBSS, 1% BSA; Invitrogen) and stimulated for 30 min at 37 °C with 100 μL FR α [25], human sera (1 : 100 dilution), NIP-BSA, or rabbit anti-IgE (200 ng/mL). To detect β -hexosaminidase, 50 μL culture supernatants diluted 1 : 1 in stimulation buffer, were transferred onto black 96-well plates with 50 μL fluorogenic substrate per well (1 mM 4-nethylumbelliferyl *N*-acetyl- β -D-glucosaminide 0.1% DMSO, 0.1% Triton X-100, 200 mM citrate, pH 4.5) and incubated for 2 h in the dark. Reactions were quenched with 100 μL per well 0.5 M Tris and plates read with a Fluostar Omega microplate reader (350 nm excitation, 450 nm emission) (BMG Labtech, Offenburg, Germany). Degranulation was expressed as a percentage of Triton X-100 release, and compared with unstimulated cells (< 10%).

Enzyme-linked immunosorbent assay to detect circulating folate receptor α

To quantify circulating FR α in human sera, 96-well plates were coated overnight at 4 °C with 2 $\mu\text{g/mL}$ mouse anti-human FR α (IgG1, R&D Systems) in 0.2 M carbonate-bicar-

bonate buffer, pH 9.4. Plates were given blocking buffer (SuperBlock buffer, 0.05% Tween-20; Perbio Science UK Ltd, Cramlington, UK) for 2 h at room temperature, washed 4 $\times$ with PBS, 0.05% Tween-20 (washing buffer) and given recombinant FR α [25] or sera (1 : 5 in blocking buffer) and incubated for 2 h at room temperature. All conditions were tested in triplicate. Plates were washed 4 $\times$, incubated with 50 ng/mL goat anti-FR α -biotin in blocking buffer (R&D Systems) for 2 h at room temperature, washed again 4 $\times$, and given 50 ng/mL streptavidin-HRP (Pierce, Thermo Fisher Scientific, Cramlington, UK) in blocking buffer for 30 min at room temperature. Following five washes, samples were treated with 0.5 mg/mL *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich, Gillingham, UK) in peroxide substrate buffer (Pierce) for 5 min at room temperature. Following addition of 50 μL per well stop solution (1 M HCl), plates were read at 492 nm (reference wavelength 650 nm) on a Fluostar Omega microplate reader. The lower limit of quantification of FR α in this assay was 1.25 ng/mL.

Enzyme-linked immunosorbent assay to detect circulating anti-folate receptor α immunoglobulin G autoantibodies

To quantify anti-FR α IgG autoantibodies in human sera, 96-well plates were coated overnight at 4 °C with 1 $\mu\text{g/mL}$ recombinant human FR α (R&D Systems) in 0.2 M carbonate-bicarbonate buffer, pH 9.4. Plates were incubated in blocking buffer (SuperBlock buffer, 0.05% Tween-20; Perbio Science UK Ltd) for 2 h at room temperature, washed 4 $\times$ with PBS, 0.05% Tween-20 (washing buffer) and incubated with MOv18 IgG or human sera (1 : 5 in blocking buffer) for 2 h at room temperature. All conditions were tested in triplicate. Plates were washed 4 $\times$, and incubated with 160 ng/mL F(ab')₂ goat anti-human Fc γ -HRP (Jackson ImmunoResearch, Stratech, Newmarket, UK) in blocking buffer (Stratech) for 45 min at room temperature. Following five washes, samples were treated with 0.5 mg/mL *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) in peroxide substrate buffer (Pierce) for 5 min at room temperature. Following addition of 50 μL per well stop solution (1 M HCl), plates were read at 492 nm (reference wavelength 540 nm) on a Fluostar Omega microplate reader. The lower limit of quantification of anti-FR α IgG in this assay was 2.5 ng/mL.

Whole blood basophil activation test

Activation of human basophils *ex vivo* was measured using a standard BAT, which measures up-regulation of CD63 on (CCR3^{high}SSC^{low}) basophils in whole human blood as described previously [31, 32]. Briefly, blood collected in 1.8 mg/mL K₂EDTA was assayed using a FLOW 2 CAST basophil activation kit (Bühlmann, Schönenbuch, Switzerland) and acquired within 4 h on a dual laser FACSCanto (BD Biosciences, Oxford, UK). To 100 μL blood were added 150 μL of stimulation buffer alone

(background), or 100 μ L 'stimulus' with 50 μ L stimulation buffer. Stimuli included positive controls, e.g. 1 μ g/mL anti-human Fc ϵ R1 polyclonal or anti-human IgE polyclonal antibodies, or MOv18 IgE (ranges from 0.03 to $\sim$ 10 μ g/mL). Some blood samples pre-incubated with MOv18 IgE were given 100 μ L human recombinant FR α solution (8–333 ng/mL) and incubation continued for a further 10 min. Samples were finally incubated with staining reagent (0.1 μ g/mL mouse anti-human CCR3-PE and CD63-FITC antibodies) at 37 °C for 10 min before acquisition of at least 500 basophil (CCR3^{high}SSC^{low}) events per sample.

Live cell imaging

IGROV-1 tumour cells were seeded at 2×10^4 cells/well onto 12-well glass-bottomed plates (MatTek, Ashland, MA, USA) and labelled with 500 μ L of 2 μ M Calcein AM per well (Molecular Probes, Invitrogen) for 30 min, washed and resuspended in RPMI 1640 medium, with 10% FCS. RBL SX-38 cells (2×10^5 /mL) were labelled for 30 min at 37 °C with 2 μ g/mL Cholera Toxin B subunit Alexa Fluor[®] 647 (Invitrogen), which binds ganglioside 1 (GM1), and is a marker of granule cycling [33, 34]. Cells were washed and treated with MOv18 IgE or NIP IgE in medium for 30 min. The adherent labelled tumour cells and 4×10^4 RBL SX-38 cells/well were incubated for 30 min at 37 °C in a humidified chamber. Images were captured using a Zeiss AxiovertZ.1 microscope (Welwyn Garden City, UK) (with incorporated Calibri System) equipped with a Plan-Apochromat 63 $\times$ /1.4 oil DIC objective, Axio CamMR3 camera and AxioVision software system (Carl Zeiss). Measurements of mean fluorescence intensity per cell (MFI $\pm$ SD, five image fields per condition) were conducted using ImageJ version 1.4 image processing and analysis software (<http://rsbweb.nih.gov/ij/index.html>).

Statistical methods

A two-tailed Student's *t*-test was used to compare % RBL SX-38 cell degranulation and FR α concentrations in patient and healthy control sera (values $P < 0.05$, were considered statistically significant). Linear regression analysis was used to assess correlation between degranulation with MOv18 IgE and FR α titres in patient serum samples. Statistical analyses were performed using GraphPad Prism version 5.03.

Results

MOv18 immunoglobulin E and soluble antigen do not trigger effector cell degranulation

We explored whether MOv18 IgE triggers Fc ϵ R1-mediated degranulation of RBL SX-38 cells, a validated model of human Fc ϵ R1 cross-linking in the presence of specific IgE and polyvalent allergens (Fig. 1a) [28, 35, 36]. Flow cytometric analysis confirmed that MOv18 IgE bound to

almost 100% of the human Fc ϵ R1-bearing cells, and that FR α in turn bound to virtually all of the IgE-primed cells (Fig. 1b). Cellular degranulation was measured by β -hexosaminidase release expressed as a percentage of that measured with Triton X-100 detergent (complete cell lysis). Unstimulated and NIP IgE-stimulated cells triggered background degranulation ($< 10\%$ of Triton X-100, Fig. 1c). NIP IgE with its multivalent antigen NIP BSA or with polyclonal anti-IgE cross-linking Fc ϵ R1-bound IgE, triggered detectable degranulation (mean $\pm$ SD, $34.5 \pm 4.1\%$, $25.4 \pm 2.9\%$, respectively, $n = 3$) [37].

We then investigated whether MOv18 IgE alone or in the presence of soluble FR α can trigger degranulation. As with NIP IgE, MOv18 IgE alone did not induce degranulation ($1.5 \pm 1.1\%$), but MOv18 IgE cross-linked by polyclonal anti-IgE antibody induced appreciable degranulation ($37.6 \pm 6.7\%$). Degranulation was also triggered through cross-linking of cell surface MOv18 IgE-bound FR α by an anti-FR α polyclonal antibody ($15.4 \pm 0.7\%$) to mimic the effect of a multimeric antigen or of tumour cells bearing multiple copies of the antigen. When cells were incubated with MOv18 IgE and increasing concentrations of recombinant FR α (1.9–1000 ng/mL) we measured background degranulation, even at supra-physiological concentrations (> 100 ng/mL) and concentrations > 10 -fold (400–1000 ng/mL) in excess of those reported in ovarian cancer patient blood [39] (Fig. 1d).

MOv18 immunoglobulin E does not stimulate degranulation in the presence of sera from ovarian carcinoma patients or healthy volunteers, despite detectable circulating folate receptor α

We examined whether MOv18 IgE-primed RBL cells showed evidence of Fc ϵ R1-dependent activation in the presence of naturally shed, circulating FR α in sera from patients with ovarian carcinoma. Sera from 32 patients with stage III or IV disease caused minimal degranulation which was not statistically different to that observed in the presence of 14 normal control sera ($2.45 \pm 1.53\%$ vs. $3.05 \pm 2.40\%$, $P > 0.05$; Table 1, Fig. 2a) despite the fact that the median FR α concentration in the patients' sera was clearly and significantly elevated compared with that measured in the normal sera ($P = 0.006$, Fig. 2b). No significant differences in degranulation were measured in relation to disease stage: sera from patients with stage III disease ($n = 20$) compared with sera from patients with stage IV ovarian carcinoma ($n = 12$, $P > 0.05$). We measured very low FR α levels in 14 healthy volunteer sera (1.73 ng/mL) and FR α titres above the mean in the healthy volunteer cohort in 19 out of 30 ovarian carcinoma patient sera (5.48 for stage III, 9.59 ng/mL for stage IV groups; total mean 7.1 ng/mL, Table 1). What little degranulation was observed in the presence of patient sera did not correlate with the disease stage or absolute serum concentration of FR α (Fig. 2c).

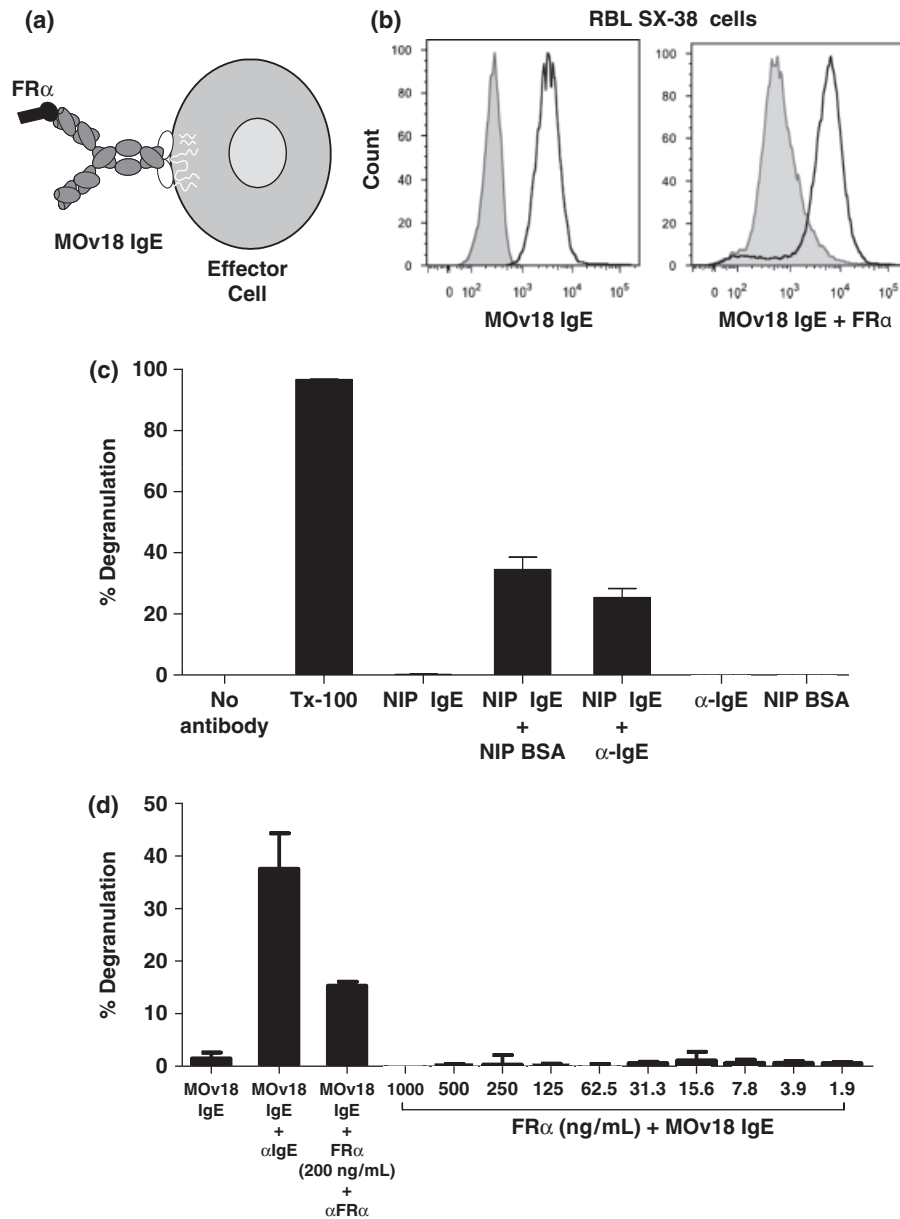


Fig. 1. MOv18 IgE and soluble $FR\alpha$ bind to RBL SX-38 cells and do not stimulate degranulation. (a) Schematic diagram of the monovalent interaction between shed $FR\alpha$ and MOv18 IgE on the surface of effector cells. (b) Flow cytometric assay demonstrating MOv18 IgE binding to RBL SX-38 cells (solid line histogram: MOv18 IgE; grey histogram: goat anti-IgE-FITC antibody control); and $FR\alpha$ binding to MOv18 IgE-primed RBL SX-38 cells (solid line histogram: MOv18 IgE+ $FR\alpha$ +goat anti-human $FR\alpha$ +donkey anti-goat-Alexa Fluor[®] 448 antibodies; grey histogram: MOv18 IgE+goat anti-human $FR\alpha$ +donkey anti-goat-Alexa Fluor[®] 448 antibodies). (c) β -hexosaminidase release showing % degranulation relative to Triton X-100. Minimal degranulation was seen with cells alone, control antibody NIP IgE, its polyclonal ligand NIP-BSA, or polyclonal anti-IgE antibody alone. Significant degranulation is observed with samples given NIP IgE+NIP-BSA or NIP IgE+anti-IgE. (d) Degranulation with MOv18 IgE plus anti-IgE and minimal degranulation with MOv18 IgE alone and MOv18 IgE plus recombinant $FR\alpha$ (1.9–1000 ng/mL). Scale bars indicate mean values $\pm$ standard deviation [38] of $n = 3$ experiments, all conditions were tested in triplicate. $FR\alpha$, folate receptor α ; RBL, rat basophilic leukaemia; BSA, bovine serum albumin.

Immunoglobulin E and tumour cells trigger degranulation in an antigen-dependent manner

A large proportion of ovarian carcinoma cells over-express $FR\alpha$ on their cell surface. MOv18 IgE may bridge effector and tumour cells, triggering $Fc\epsilon R1$ cross-linking through multiple copies of cell surface $FR\alpha$ (Fig. 3a). To

explore the consequences of this interaction, RBL SX-38 cells were sensitized with MOv18 IgE and incubated with serially increasing numbers of $FR\alpha$ -expressing IGROV-1 ovarian carcinoma cells (Fig. 3b). We were able to detect significant degranulation at higher IGROV-1 : RBL SX-38 cell ratios ($> 15 : 10$), but not at the lower ratios ($< 8 : 10$) which one might expect to find in the peripheral blood of

Table 1. Percent RBL SX-38 cell degranulation in the presence of MOv18 IgE and measurement of serum FR α levels in human sera from (A) healthy volunteers and (B) ovarian carcinoma patients

Volunteer no.	Serum origin			% Degranulation		[FRα] (ng/mL)
(A) Healthy volunteers						
1		Healthy			2.68	ND
2		Healthy			0.99	2.30
3		Healthy			1.10	ND
4		Healthy			0.97	ND
5		Healthy			2.78	10.01
6		Healthy			1.66	ND
7		Healthy			2.13	2.74
8		Healthy			3.34	ND
9		Healthy			N/A	ND
10		Healthy			3.65	ND
11		Healthy			1.91	ND
12		Healthy			2.64	9.15
13		Healthy			9.43	ND
14		Healthy			6.38	ND
					Mean±SD	Mean [FRα]±SD
					3.05±2.40	1.73±3.45
Volunteer no.	Serum origin	Stage	Histological subtype	Primary treatment/relapse	% Degranulation	[FRα] (ng/mL)
(B) Ovarian carcinoma patients						
15	Patient	3	PD serous	Primary	1.44	5.18
16	Patient	3	Serous	Primary	3.27	ND
17	Patient	3	Serous	Primary	3.17	21.01
18	Patient	3	Papillary serous	Primary	3.92	N/A
19	Patient	3	Serous	Primary	1.27	8.27
20	Patient	3	PD serous	1st relapse	1.38	ND
21	Patient	3	Serous adeno	1st relapse	1.66	2.58
22	Patient	3	PD serous	1st relapse	1.40	5.62
23	Patient	3	Papillary serous	1st relapse	3.22	ND
24	Patient	3	Serous adeno	1st relapse	3.55	6.82
25	Patient	3	PD serous	1st relapse	1.29	5.50
26	Patient	3	WD serous	1st relapse	3.01	14.90
27	Patient	3	Serous adeno	1st relapse	3.14	1.60
28	Patient	3	Serous fallopian	1st relapse	1.85	15.85
29	Patient	3	Not known	1st relapse	1.68	ND
30	Patient	3	Primary Peritoneal	1st relapse	1.24	6.30
31	Patient	3	Not known	1st relapse	1.40	ND
32	Patient	3	Not known	1st relapse	2.24	1.53
33	Patient	3	PD serous	1st relapse	2.57	3.45
34	Patient	3	PD serous	1st relapse	2.41	N/A
					Mean±SD	Mean [FRα]±SD
					2.26±0.90	5.48±6.14
35	Patient	4	Clear cell	5th relapse	2.47	12.67
36	Patient	4	Serous	4th relapse	4.04	1.59
37	Patient	4	MD serous	1st relapse	0.0	14.26
38	Patient	4	PD adeno	5th relapse	8.70	ND
39	Patient	4	Adeno	2nd relapse	2.50	21.01
40	Patient	4	PD adeno	3rd relapse	2.40	2.50
41	Patient	4	Pap serous	Primary	1.60	ND
42	Patient	4	Serous	Primary	3.70	5.64*
43	Patient	4	Clear cell	1st relapse	2.76	34.45*
44	Patient	4	Serous	1st relapse	2.85	11.84*
45	Patient	4	Adeno	2nd relapse	2.14	9.67*
46	Patient	4	Clear cell	1st relapse	0.0	1.49*

Table 1. continued

Volunteer no.	Serum origin	Stage	Histological subtype	Primary treatment/relapse	% Degranulation	[FR α] (ng/mL)
					Mean $\pm$ SD	Mean [FR α] $\pm$ SD
					2.76 $\pm$ 2.24	9.59 $\pm$ 10.29
					Overall mean $\pm$ SD	Overall mean [FR α] $\pm$ SD
					2.45 $\pm$ 1.53	7.12 $\pm$ 8.15

*Patients tested in whole blood basophil activation assay (Fig. 5).

ND, not detected, depicting [FR α] values below 1.5 ng/mL; N/A, not analysed; PD, poorly differentiated; WD, well differentiated; MD, moderately differentiated; FR α , folate receptor α .

patients with ovarian cancer (equivalent to $<8 \times 10^3$ tumour cells/ 10^4 effector cells, typical basophil counts per mL blood) (Fig. 3b; supporting information Table S1) [34, 40–45]. Tumour cells that did not express FR α did not induce degranulation, even at high tumour:effector cell ratios (6:1), suggesting that the phenomenon is antigen-specific.

We confirmed that RBL SX-38 cells (red) were activated in the presence of MOv18 IgE and tumour cells using live cell imaging (Fig. 3c) to monitor membrane motility and exocytosis after labelling of cell membranes with cholera toxin B subunit [33, 34]. We observed enhanced mobilization of cytoplasmic granules (reduction of red cytoplasmic fluorescence) by RBL SX-38 cells incubated with MOv18 IgE after 15 and 30 min in culture (depicted by reduction in mean fluorescence intensity per cell, MFI $\pm$ SD, measured at 58.3 $\pm$ 26.4% and 36.6 $\pm$ 17.2% of original after 15 and 30 min respectively, $n=5$) compared with samples treated with control NIP IgE antibody (91.4 $\pm$ 15.5% and 77.5 $\pm$ 14.9%, $n=5$, $P=0.028$).

Examination of early signals of blood basophil activation ex vivo following administration of MOv18 immunoglobulin E by the basophil activation test assay

To determine whether MOv18 IgE activates blood basophils from patients with ovarian carcinoma directly *ex vivo* we used the BAT using CD63 as a readout of basophil activation [31, 46]. Figure 4 summarizes a typical set of experiments in a healthy control. Human basophils (CD123^{high}SSC^{low}) bound MOv18 IgE (CD123^{high}IgE⁺), and basophils (CCR3^{high}SSC^{low}) expressing CD63 (CCR3^{high}CD63⁺) were detected in the presence of stimuli (Figs 4a and b). Only 2.3% of unstimulated basophils expressed CD63, in agreement with previous reports [32]. This increased to 81.48% and 89.58% following cross-linking of Fc ϵ RI with either polyclonal anti-Fc ϵ RI (Fig. 4b) or anti-IgE (Figs 4c and d) antibodies respectively. In contrast a range of concentrations of MOv18 alone (0.03–3.5 μ g/mL) did not augment CD63 expression (Figs 4c and d).

We further investigated whether MOv18 IgE and soluble FR α can trigger basophil activation in whole human blood

from healthy volunteers. In the presence of MOv18 IgE, addition of exogenous FR α even at concentrations 10-fold higher than those observed in patients (333 ng/mL, $n=6$) did not augment CD63 expression (Figs 5a and b). Similarly, addition of MOv18 IgE to blood of five patients with ovarian carcinoma did not result in detectable augmentation of CD63 expression (Figs 5c and d) despite the fact that all five patients had detectable circulating concentrations of FR α (Table 1). In contrast, cross-linking with anti-Fc ϵ RI or anti-IgE polyclonal antibodies clearly augmented CD63 expression (supporting information Figure S1).

We also examined sera from 24 patients in which we had detected the presence of circulating FR α antigen (Table 1) for the presence of anti-FR α IgG autoantibodies that might crosslink sFR α bound to MOv18 IgE-sensitized basophils. We found no detectable increase above background in sera from 18 of these patients. In the remaining six patients and we detected concentrations in the range of 3.23–42.64 ng/mL (supporting information Table S2). When tested in the RBL SX-38 assay, sera from these patients did not trigger any functional degranulation in the presence of MOv18 IgE. Further, in two patients with detectable autoantibodies, we could detect no increase in CD63 expression by the patients' blood basophils using the BAT.

Discussion

The human immune system uses five classes of antibodies to combat disease. Each antibody class operates in different anatomical compartments, and functions through unique Fc-receptors and immune effector cells. Understanding how antibody class influences the nature of the immune response harnessed against cancer cells may be key in the design of future effective antibody therapeutics. Research to-date has mostly focused on developing IgG antibodies, the most abundant circulating antibody class, and all antibodies currently licensed for cancer therapy are IgG. We have previously shown, using ovarian xenograft models in immunodeficient mice, that survival following treatment with the anti-FR α chimeric antibody MOv18 IgE with human PBMC as effector cells is superior to that with MOv18 IgG1 [2–5, 7]. This suggests that harnessing IgE as a therapy for human cancer may

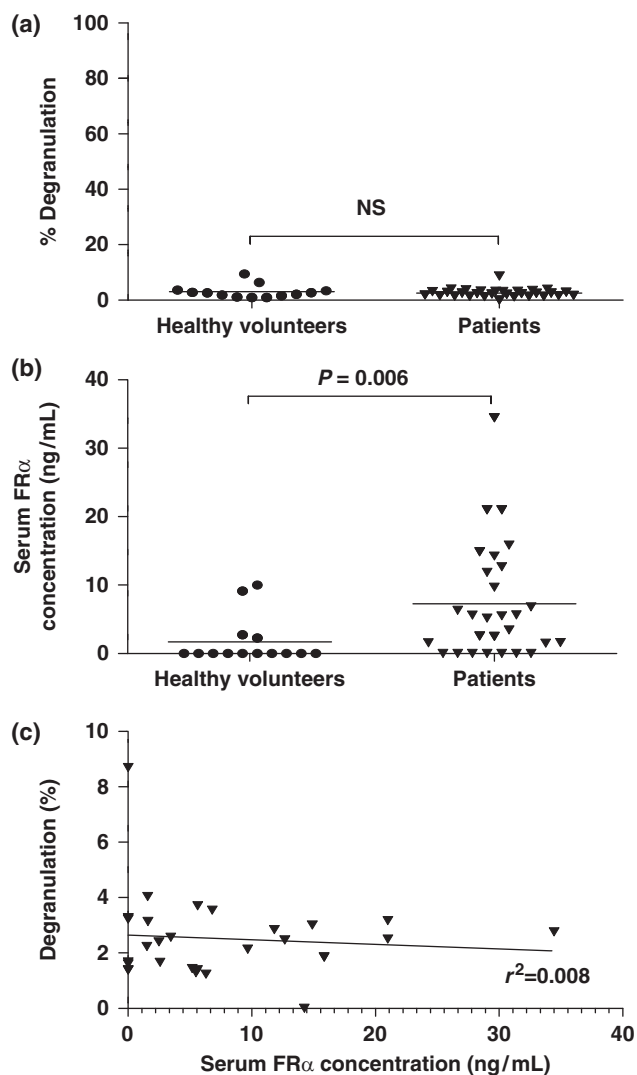


Fig. 2. Sera from ovarian carcinoma patients do not stimulate MOv18 IgE-primed RBL SX-38 cell degranulation despite elevated FR α concentrations. (a) β -hexosaminidase release assays performed with 14 healthy volunteer and 32 ovarian carcinoma patient sera; degranulation was minimal in both groups with no significant differences observed between groups ($P > 0.05$). (b) Serum samples from the above cohort were quantified by ELISA: [FR α] increased in ovarian carcinoma patients compared with healthy volunteers ($P = 0.006$). (c) No correlation ($r^2 = 0.008$) is seen between % degranulation and serum [FR α] in the ovarian carcinoma patient cohort. NS, not significant; FR α , folate receptor α ; RBL, rat basophilic leukaemia.

provide increased efficacy compared with IgG-based therapies. However, before this hypothesis can be tested in patients, it is important to demonstrate that this approach is not likely to trigger type I hypersensitivity.

In the present study we made use of the RBL SX-38 cell line to examine human Fc ϵ RI-mediated effector activity through recognition of MOv18 IgE and its antigen FR α , excluding variability associated with the use of human mast cells and basophils. These are difficult to obtain in sufficient purity and numbers and have to be stripped of

bound endogenous IgE, an aggressive procedure. In contrast, RBL cells express pristine, unoccupied, human tetrameric Fc ϵ RI which can be saturated with IgE antibodies of interest. While in theory RBL cells may differ physiologically from human cells, in practise they have shown high sensitivity and specificity for detecting the capacity of various allergens to induce type I hypersensitivity after sensitization by IgE from the serum of allergic human subjects using release of the granule protein β -hexosaminidase as a readout [28, 35, 48, 49].

We have shown that MOv18 IgE in the presence of recombinant FR α antigen does not trigger degranulation in this system, whereas cross-linking of Fc ϵ RI with an anti-Fc ϵ RI antibody or an antibody recognizing MOv18 IgE-bound FR α on the surface of RBL SX-38 cells does trigger degranulation. These data suggest that once cross-linked, MOv18 IgE-FR α complexes can mediate effector cell activation, while FR α is inactive in its monomeric form. To account for any effects on effector cell activation mediated by circulating FR α antigen released from tumour cells of patients, we measured serum concentrations of FR α by ELISA. In agreement with previous studies [39], we measured increased circulating antigen in patients compared with healthy volunteers. Nevertheless, MOv18 IgE did not trigger degranulation of the readout cells in the presence of sera from either cohort. These findings are consistent with the hypothesis that soluble FR α antigen binds to, but does not cross-link surface-bound MOv18 IgE. The presence of patient serum also takes some account of effects of other circulating agents that may potentially cross-link Fc ϵ RI such as endogenous anti-FR α autoantibodies, which we detected in sera of a subset of patients. Nevertheless, our admittedly somewhat limited data so far showed no evidence that these antibodies triggered activation of the relevant readout cells.

In an *ex vivo* setting, MOv18 IgE similarly did not induce early signs of basophil activation in the blood of patients with ovarian carcinoma, even at concentrations of 10 μ g/mL, which we estimate as equivalent to a clinical therapeutic dose of 1 mg/kg. Here, we used the BAT tool, an emerging assay which has demonstrated sensitivity and specificity in the clinical diagnosis of type I hypersensitivity to a range of allergens including venom, latex, food allergens and drugs [31]. A caveat with these experiments is that the responder basophil Fc ϵ RI receptors are likely to be partly occupied with endogenous IgE *ex vivo*, allowing only partial occupancy by MOv18 IgE. On the other hand, these experiments take further account of any additional factors found in whole blood (but removed in the isolation of serum), that could trigger IgE-mediated basophil activation, such as circulating immune effector cells and, in cancer patients' blood, circulating tumour cells or fragments.

Although quantification of circulating tumour cells in patients with ovarian cancer is not standardized, figures in

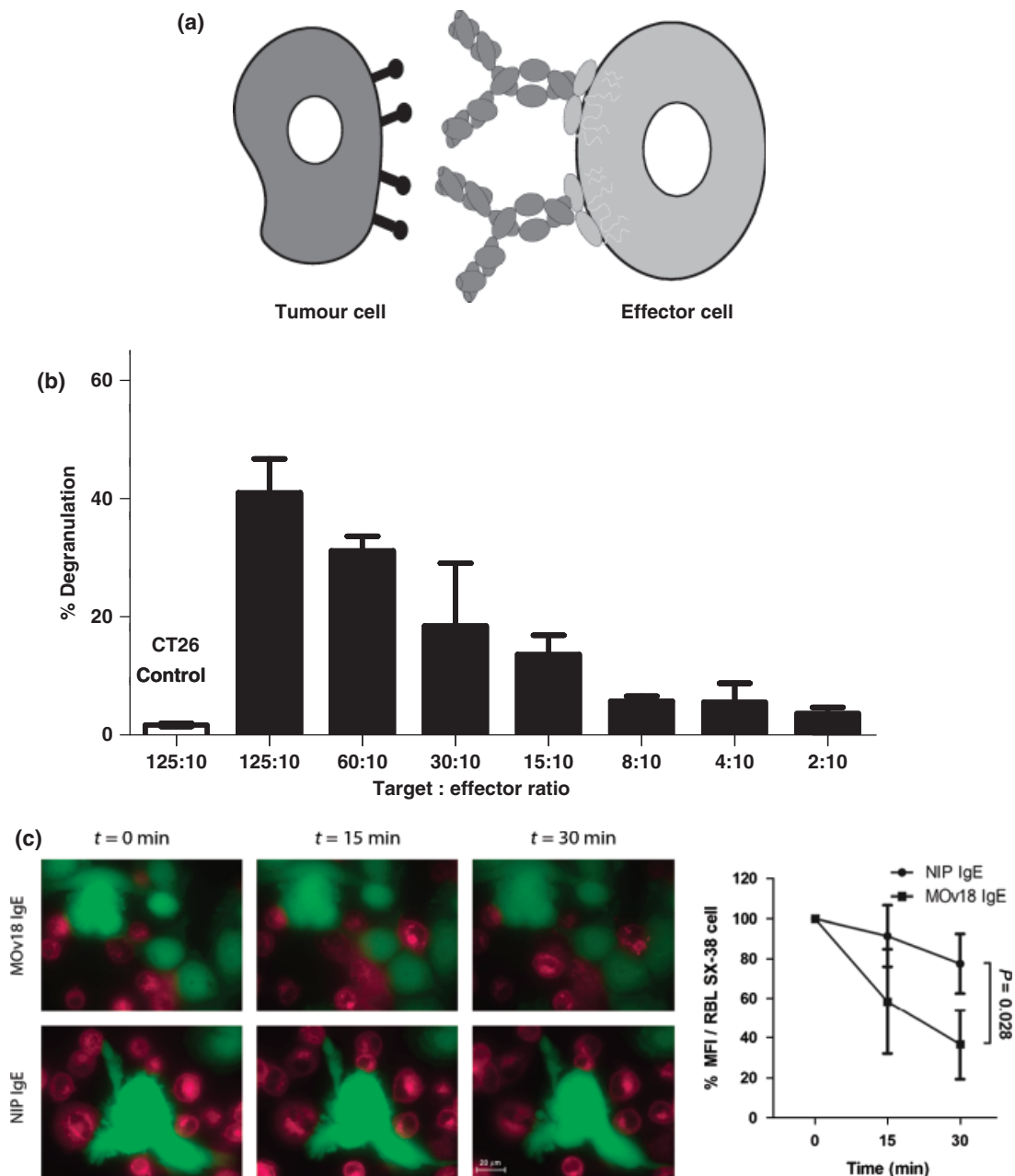


Fig. 3. MOv18 IgE stimulates RBL SX-38 cell degranulation in the presence of increasing numbers of IGROV-1 tumour cells. (a) Schematic diagram of FR α -expressing tumour cell cross-linking Fc ϵ RI-bound MOv18 IgE on mast cells. (b) Degranulation of RBL SX-38 mast cells treated with MOv18 IgE in the presence of increasing numbers of IGROV-1 tumour cells. Significant degranulation was detected with increasing Target:Effector cell ratios ($> 15 : 10$). Minimal degranulation is seen with non-FR α -expressing tumour cells (CT26). Scale bars indicate mean values $\pm$ standard deviation [38] of $n = 3$ experiments, all conditions were tested in triplicate. (c) Left: Representative images of RBL SX-38 cell (labelled with Cholera Toxin subunit B, red) membrane mobilization in the presence of IGROV-1 tumour cells (labelled with Calcein AM, green) with MOv18 IgE (top panel) or control NIP IgE antibody (bottom panel) following 0, 15 and 30 min at 37 °C (magnification $\times 63/1.4$ oil DIC objective, scale bars: 20 μ m). Right: Quantitative assessments of % mean fluorescence intensity (MFI $\pm$ SD) per cell for Cholera Toxin subunit B-labelled RBL SX-38 cells (red fluorescently labelled cells from left), measured after 15 and 30 min with either MOv18 IgE or control NIP IgE antibodies ($n = 5$), showing reduced % MFI for MOv18 IgE cells ($P = 0.028$), which depicts enhanced membrane mobilization in the presence of antigen-specific antibody and tumour cells. FR α , folate receptor α ; RBL, rat basophilic leukaemia.

the range of 0–566 cells/mL have been reported [40, 41, 43–45], except for one report of a patient with as many as 3118 tumour cells/mL [42]. Our data show that even at a tumour cell burden of 4000 cells/mL (a density above any

reported in cancer patient blood), MOv18 IgE induced minimal Fc ϵ RI-mediated activation of readout cells (Fig. 3), and did not augment CD63 expression on basophils in the patients' whole blood (Fig. 5). It is possible

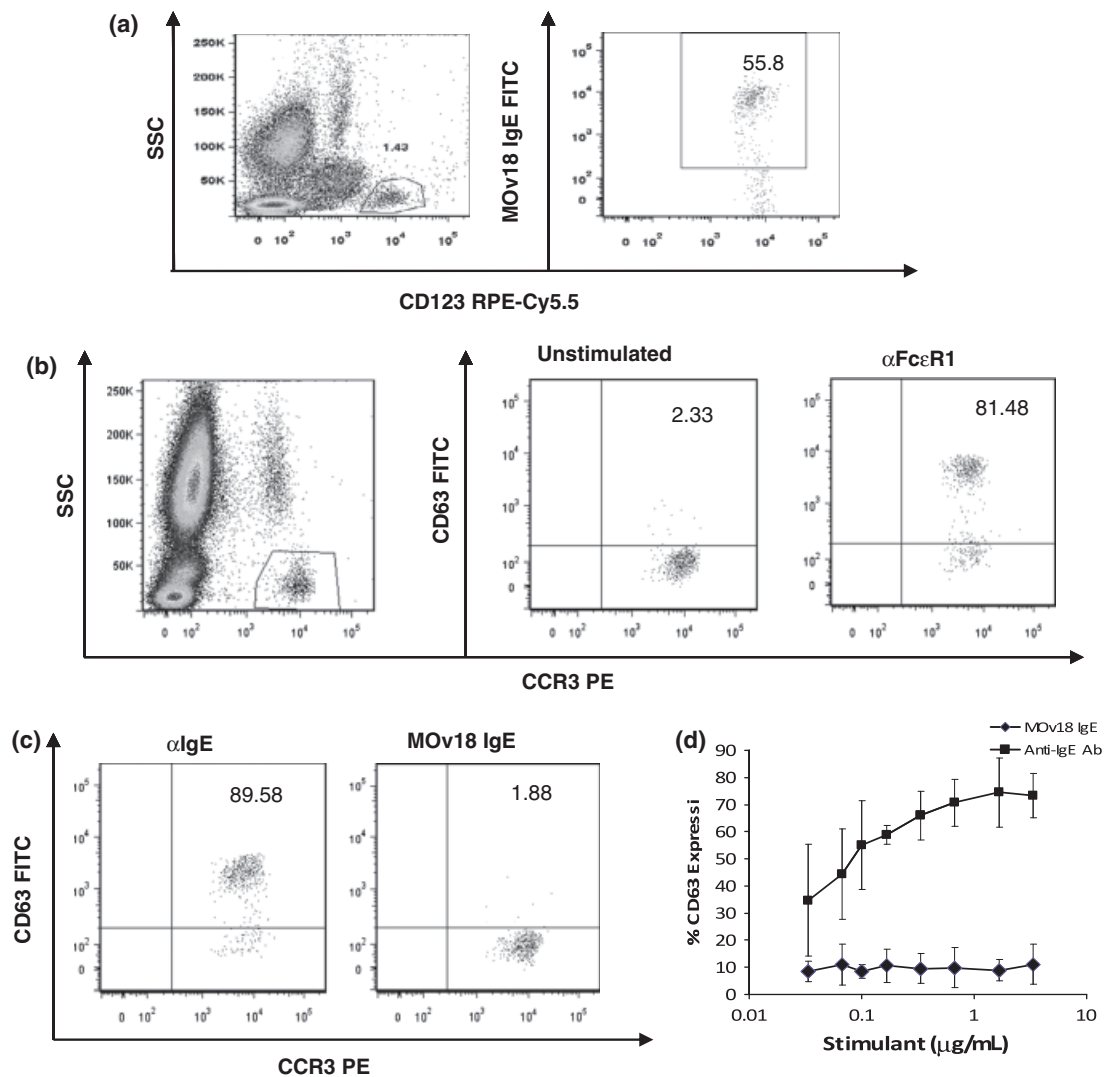


Fig. 4. MOV18 IgE does not trigger CD63 expression in whole human blood. (a) Flow cytometric dot plots identify basophils in whole blood (CD123^{high}SSC^{low}, left), and basophils bind MOV18 IgE (CD123^{high}IgE⁺, right). Basophil activation assessments with representative two-colour dot plots of: (b) % CCR3^{high}SSC^{low} basophils (gated, left) expressing CD63 in unstimulated blood (CCR3^{high}CD63⁺, middle), and with anti-Fc ϵ R1 polyclonal antibody [47]; (c) Increased % CCR3^{high}CD63⁺ basophils with anti-IgE polyclonal antibody (left), but not with MOV18 IgE (0.7 μ g/mL, right). (d) % CCR3^{high}CD63⁺ basophils in whole blood titrated with either MOV18 IgE or anti-IgE polyclonal antibody (0.03–3.5 μ g/mL). Scale bars indicate mean values $\pm$ standard deviation [38] of $n = 5$ experiments, all conditions were tested in triplicate.

that, in some MOV18-treated patients antibody-induced tumour cell death could release fragments of tumour cells from lesions that might activate basophils in the periphery. On the other hand our previous studies [2–4] suggest that MOV18 IgE triggers not only cytotoxicity, but also substantial tumour cell infiltration and phagocytosis by monocytes/macrophages that may limit their escape into the circulation. We will monitor this in patients treated with MOV18 IgE by repeated skin prick and intradermal testing with the antibody before each dosage by monitoring serum concentrations of FR α , β -tryptase and by repeated basophil activation testing with MOV18 in the patient's own whole blood.

At high tumour cell concentrations, where the density of epitope arrayed on the tumour cell surface may stimulate effector cells through IgE cross-linking, we did observe significant Fc ϵ R1-mediated activation of readout cells (Fig. 3). We speculate that this may resemble the situation where tumour deposits are infiltrated by effector cells. Infiltration of some tumours by mast cells is well described [20, 21]. This may trigger IgE-specific anti-tumour immune mechanisms in the resident mast cells and possibly infiltrating basophils, as we have previously observed for monocytes and eosinophils [2, 3, 6, 7]. We are aware that a number of studies with other cancers have suggested that the presence of mast cells and other

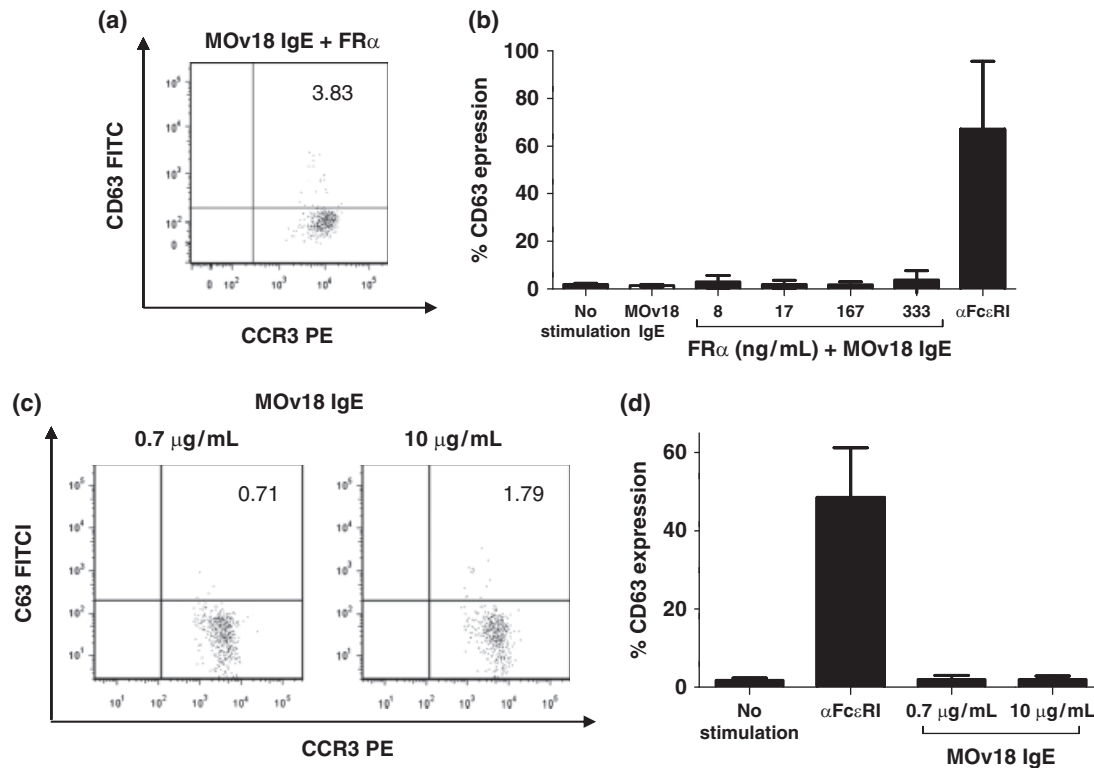


Fig. 5. MOv18 IgE does not trigger CD63 expression by blood basophils in the presence of soluble monomeric antigen or in the blood of patients with ovarian carcinoma. (a) Representative two-colour flow cytometric dot plot of % CCR3^{high}CD63⁺ basophils exposed to MOv18 IgE and human FR α (17 ng/mL) showing background CD63 expression. (b) Basophil activation did not increase in blood from healthy volunteers treated with MOv18 IgE and increasing concentrations of FR α , but efficient activation occurs with anti-Fc ϵ RI control. Scale bars indicate mean values $\pm$ standard deviation [38] of $n = 6$ experiments, all conditions were tested in triplicate. (c) Representative two-colour flow cytometric dot plots of % CCR3^{high}CD63⁺ basophils in patient blood given MOv18 IgE (0.7 and 10 μ g/mL). (d) % CCR3^{high}CD63⁺ basophils in blood of a patient cohort ($n = 5$) with detectable serum FR α titre (indicated in Table 1), illustrating background expression with addition of MOv18 IgE compared with anti-Fc ϵ RI and no stimulation controls. Scale bars indicate mean values $\pm$ standard deviation [38] of $n = 5$ experiments, all conditions were tested in triplicate. FR α , folate receptor α .

immune effector cells, such as monocytes, in tumours has been correlated with tumour progression [20, 21], and that activation of mast cells within a tumour mass may at least in theory trigger hypersensitivity *in situ*. Nevertheless the data we have so far concerning the effects of MOv18 IgE on tumour killing and progression in animal models suggest added benefits of MOv18 IgE over IgG antibodies and have not so far produced any evidence whatsoever of systemic adverse effects [2–4, 7].

Although we here report that circulating FR α in patients with ovarian cancer did not activate our readouts of hypersensitivity, another potentially detrimental effect of circulating FR α is that it might bind to MOv18 IgE bound to cytotoxic effector cells, blocking the ability of these cells to recognize tumour cell-associated FR α antigen. Our *in vitro* assays suggest that soluble FR α at 200 ng/mL may saturate cell surface-bound MOv18 IgE on cytotoxic effector cells, but this greatly exceeds concentrations we have so far observed in patients with advanced ovarian carcinoma (Fig. 2). Thus, we would expect that most of the cell-bound MOv18 IgE will be unoccupied and able to bind tumour cell surface-bound FR α in most, but perhaps

not in all of the patients. This concern has been examined in the context of treatment with trastuzumab, where some patients with HER2/*neu*+ carcinomas have circulating serum soluble HER2 receptor concentrations exceeding 15 ng/mL, which was postulated potentially to reduce the efficacy of trastuzumab. Clinical experience with trastuzumab has however uncovered no clear correlation between serum HER2 concentrations and clinical responsiveness [50]. We will examine this question in future clinical studies with MOv18 IgE by measuring Fc ϵ RI-MOv18 IgE occupancy by circulating FR α on immune effector cells and by correlating serum FR α concentrations with clinical efficacy in dose escalation studies.

Despite being reassured by the fact that patient sera and whole blood did not activate our hypersensitivity readouts in the presence of MOv18 IgE we were intrigued by the possibility that these patients might make IgG autoantibodies against FR α and therefore developed an ELISA assay to measure these. We did indeed detect low concentrations of these autoantibodies in a small subset of our patients. Given, however that blood and sera from a

selection of these same patients did not activate our hypersensitivity readouts, and also the fact that IgG anti-folate receptor autoantibodies at similar or higher titres than those of our patients have been described in normal individuals and patients with a history of neural tube defect pregnancies, infertility and oral cleft defects [51], we are not sure of the pathophysiological significance, if any, of this observation. Obviously, because of the theoretical risk that such antibodies could cross-link MOv18 IgE-bound FR α on cells such as mast cells and basophils, we will be monitoring the prevalence and evolution of these antibodies in treated patients in addition to the precautions referred to above. We will also, as is routine in studies with biologicals, be monitoring possible antibody responses to MOv18 IgE itself.

Systemic anaphylaxis is the most extreme manifestation of Fc ϵ RI-mediated type I hypersensitivity. Very little is known about what predisposes IgE-sensitized individuals to systemic anaphylaxis. Conventional wisdom is that systemic anaphylaxis is caused by mast cell mediators because episodes are often accompanied by transient increases in serum histamine and beta-tryptase. There are however cases where histamine alone is elevated, interpreted by some to suggest that anaphylaxis may in some instances be caused primarily by basophil activation [52]. Insofar as no known test can predict or reliably exclude whether IgE-sensitized subjects will have systemic anaphylactic reactions to allergen exposure [53], none of the data described in the present study can address this question, although we find the lack of evidence of Fc ϵ RI-mediated effector cell activation in the presence of MOv18 IgE and its target antigen, and also in the presence of autoantibodies recognizing FR α in a subset of patients, very reassuring and sufficiently persuasive to proceed with further toxicity studies and eventual clinical studies of MOv18 therapy for ovarian carcinoma. The encouraging early safety and efficacy reports using the IgG anti-FR α antibody (farletuzumab; MORAb-003) and the chimeric MOv18 IgG1 anti-FR α antibody for ovarian carcinoma provide a strong case that FR α is a valid molecular target [15–17, 38, 54–57]. Our previous demonstration of superior efficacy of the FR α -specific antibody MOv18 IgE in comparison with its chimeric IgG1 counterpart [2, 3, 6, 7] encourages us to continue with our

pre-clinical studies which may mark a new era of improved antibody therapy for solid tumours.

Clinical relevance

Despite the clinical successes of monoclonal antibodies in cancer therapy, many solid tumours remain unresponsive to treatment. Using MOv18, an antibody recognizing the tumour antigen FR α , we previously demonstrated that substituting the Fc region of the commonly used IgG antibody class with that of IgE results in superior responses against solid tumours. IgE antibodies play a role in the allergic response and the danger of triggering anaphylaxis during treatment for cancer is not yet fully elucidated. Using two readouts of the potential for type I hypersensitivity, however, we provide initial evaluations supporting the prediction that administration of a tumour antigen-specific IgE in human blood does not induce activation of effector cells that may contribute to the onset of type I hypersensitivity. These preliminary findings contribute to the safety profile of MOv18 IgE before clinical studies, adding weight to the promise of this novel approach.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

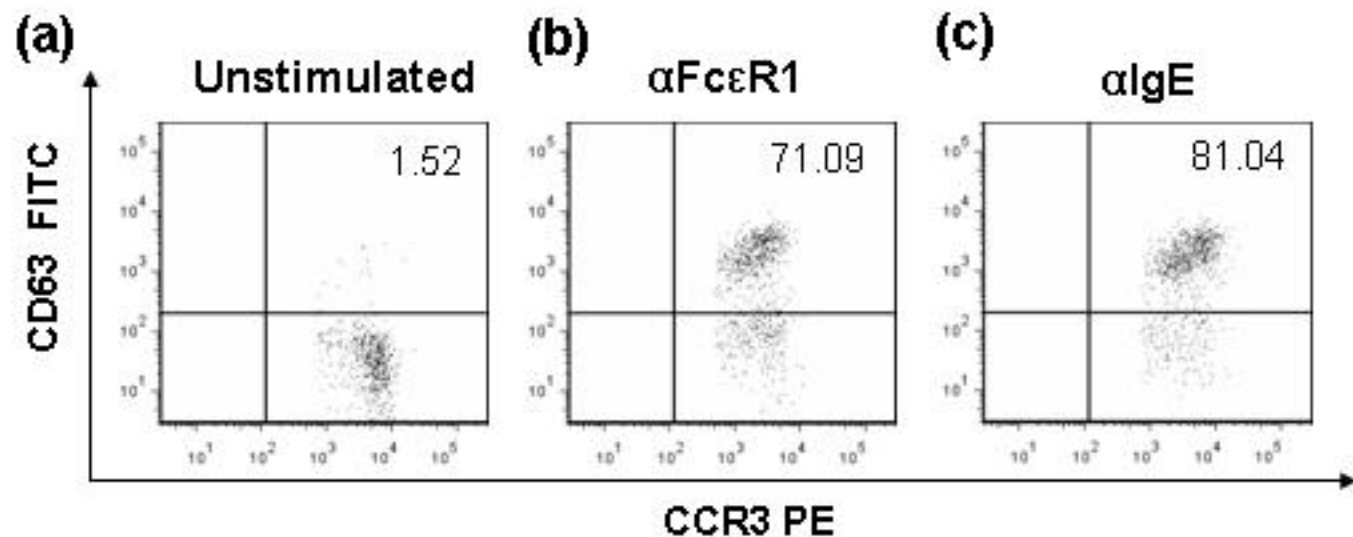
Figure S1. Two-colour flow cytometric dot plots depicting % CCR3^{high} basophils expressing CD63 in whole blood of patients with ovarian carcinoma. Examples of dot plots of % CCR3^{high}CD63⁺ events in unstimulated blood (a), and in the presence of polyclonal α -Fc ϵ RI (b) and α -IgE (c) antibodies.

Table S1. Projected % effector cell degranulation in the presence of tumour cells and IgE, based on reports of circulating tumor cell quantification in the sera of ovarian cancer patients. Calculations were projected from % degranulation measurements in Fig. 3b.

Table S2. Levels of anti-FR α IgG auto-antibodies detected by ELISA in sera from 6 ovarian carcinoma patients from a 24 cohort with detectable circulating FR α . Measurements of % cell degranulation (shown in Table 1) and % basophils expressing CD63 (basophil activation tests in Fig. 5) in the presence of MOv18 IgE are from samples shown in Table 1.

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Supplementary Figure 1



Supplementary Figure 1: Two-colour flow cytometric dot plots depicting % CCR3^{high} basophils expressing CD63 in whole blood of patients with ovarian carcinoma. Examples of dot plots of % CCR3^{high}CD63⁺ events in unstimulated blood (a), and in the presence of polyclonal α -Fc ϵ R1 (b) and α -IgE (c) antibodies.

Supplementary Table S1

No of positive †pts / no of pts assessed (% positive pts)	Max no. of *CTC / ml blood	Method of *CTC quantification	Expected Target: Effector ratio (density 10 ⁴ basophils / ml blood)	Projected Degranulation	Reference
4/4 (100%)	1.9	Ficoll Isopaque centrifugation EpCAM fluorescent antibodies	1:5,000 (0.0019:10)	< 5%	Ntouroupi <i>et al.</i> , 2008 [39]
Stages I-IV: 43/66 (60.6%)		Ficoll centrifugation Cell adhesion matrix selection			Fan <i>et al.</i> , 2009 [40]
<u>Stage I/II</u> 1/10 (10%)	6	EpCAM, ESA & pan-cytokeratins fluorescent conjugate	1:1,667 (0.006:10)	< 5%	
<u>Stage III/IV</u> 39/52 (73.1%)	149	antibodies	1:67 (0.15:10)	< 5%	
18/20 (90%)	3,118	Multiple centrifugation suspensions Anti-folate specific fluorescent antibodies	1:3.2 (3.1:10)	≤ 5%	He <i>et al.</i> , 2008 [41]
10/12 (82%)	153	Anti-folate fluorescent Ab and <i>in vivo</i> flow cytometry	1:65 (0.15:10)	< 5%	He <i>et al.</i> , 2007 [42]
31/216 (14.4%)	566	EpCAM/Cytokeratin/DAPI+ CD45 - antibodies	1:17.6 (0.566:10)	< 5%	Poveda <i>et al.</i> , 2009 [43]
Not specified (50 pts)	8	EpCAM/Cytokeratin/DAPI+ CD45 - antibodies	1:1250 (0.008:10)	< 5%	Kindelberger <i>et al.</i> , 2010 [44]
*CTC = Circulating Tumor Cells †pts = patients					

Projected % effector cell degranulation in the presence of tumour cells and IgE, based on reports of circulating tumor cell quantification in the sera of ovarian cancer patients. Calculations were projected from % degranulation measurements in Figure 3b.

Supplementary Table S2

Volunteer No.	Stage	% Degranulation	% Basophils Expressing CD63	[FRα] ng/ml	[Anti-FRα IgG] ng/ml
26	3	3.01	N/A ^{††}	14.90	26.63
32	3	2.24	N/A ^{††}	1.53	3.23
33	4	2.57	N/A ^{††}	3.45	3.33
35	4	2.47	N/A ^{††}	12.67	42.64
44	4	2.85	2.45	11.84	7.78
45	4	2.14	1.56	9.67	42.59
^{††} N/A = Not analysed					

Levels of anti-FR α IgG auto-antibodies detected by ELISA in sera from 6 ovarian carcinoma patients from a 24 cohort with detectable circulating FR α . Measurements of % cell degranulation (shown in Table 1) and % basophils expressing CD63 (basophil activation tests in Figure 5) in the presence of MOv18 IgE are from samples shown in Table 1.