Basophil activation test in cancer patient blood evaluating potential hypersensitivity to an anti-tumor IgE therapeutic candidate

To the Editor,

Monoclonal anti-tumor IgG antibodies are used widely to treat malignancies. Studies in the field of AllergoOncology, focusing on the interactions between IgE, allergy and cancer, point to biological characteristics of IgE that may engender potent anti-tumor functions[1]. These include superior affinity of IgE for cognate Fc receptors, and the presence in tumors of effector cell populations (e.g. macrophages, mast cells) known to exert anti-tumor activities when activated by IgE[2,3]. Following promising pre-clinical findings[2,4] MOv18 IgE, specific for the tumor-associated antigen folate receptor alpha (FRα), overexpressed in ovarian, basal breast cancers and other solid tumors[5], is the first anti-cancer IgE antibody studied in a first-in-class, first-in-human clinical trial (ClinicalTrials.gov Identifier: NCT02546921).

One of the potential concerns associated with application of IgE as a therapy in the clinic relates to the perceived risk of IgE-mediated anaphylaxis. Safety evaluation of such a novel agent mandated the development of bespoke methods to monitor potential hypersensitivity reactions following intravenous infusion, and ideally also to help in predicting such a reaction when selecting patients for treatment. Over the past 15 years, the basophil activation test (BAT) has been developed and widely employed to study and predict type 1 hypersensitivity reactions to food, venom and drugs in the allergy field[6,7]. Thus

far, its use in the context of cancer is limited to a small number of studies for the detection of allergic reactions to chemotherapeutic agents[8]. Basophil activation in the context of tumor-associated immunomodulation and in often heavily-treated patients has not been well-studied.

Employing the BAT in whole blood of 42 ovarian cancer patients with diverse treatment histories and tumor histologies, we examined the propensity of human basophils to be activated by anti-cancer IgE *ex vivo*. We first identified circulating basophils ($CCR3^{high}SSC^{low}$; gating strategy in Supplementary Figure A) from patients with cancer. Basophils were activated (up-regulation of CD63 expression) *ex vivo* by IgE and non-IgE-mediated triggers (anti-FcεRI, anti-IgE and fMLP, Figure 1A, Supplementary Figure B). Consistent with previously-reported findings in allergic cohorts[6], levels of basophil activation varied among individuals. We detected no basophil activation following addition of the hapten-specific NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid) IgE alone or its multivalent antigen (NIP-BSA) alone. However, we detected basophil activation by exogenous stimulation of the hapten-specific NIP IgE in combination with multimeric NIP-BSA (Figure 1A). This suggested that IgE could recognize unoccupied cell-surface FcεRI on basophils *ex vivo* and basophils could be activated by exogenous FcεRI receptor engagement and formation of cross-linking immune complexes.

We then examined whether stimulation with the anti-cancer mouse/human chimeric IgE antibody (MOv18) could trigger *ex vivo* basophil activation (Figures 1B, 1C). As expected in this cohort (n=42) stimulation with anti-FcεRI, anti-IgE and fMLP (positive controls) triggered CD63 up-regulation. In all but one patient sample, no basophil activation was measured following incubation of ovarian cancer patient blood with MOv18 IgE or control non-FRα-reactive IgE in the absence of any additional exogenous cross-linking stimulus (mean fold change in %CD63: 1.4 for MOv18 IgE, 1.3 for control IgE; 7.5 and 10.6, respectively in the positive responder) (Figure 1D). Activation, or lack thereof, was irrespective of different patient tumor histologies and treatment histories, *i.e.* a) treatment-naïve patients (n=7), b) following primary debulking surgery (n=8), c) following surgery and chemotherapy (n=21), or d) following treatment with bevacizumab (n=7) (Figures 1E, 1F). Neither MOv18 IgE nor control non-FRα-reactive IgE triggered basophil activation in the blood of a patient with already raised serum tryptase, a marker which could indicate mastocyotsis (although this clinical information was not available) and may have potentially predisposed this individual to an increased risk of hypersensitivity to IgE stimulation, including to MOv18 IgE (Figure 1G).

Since MOv18 IgE recognizes the tumor-associated antigen, FRα, it is possible that FRα shed from cancer cells in tissues and anti-FRα autoantibodies (autoAbs), if present in patient circulation, could form immune complexes with MOv18 IgE. This may result in FcεRI cross-linking and basophil activation (Figure

2A). No CD63 up-regulation on basophils was measured following *ex vivo* stimulation with either MOv18 IgE or control IgE in any sample from patients with known tumor FRα expression status, as determined by immunohistochemistry (Figures 2B, 2C, Supplementary Table). Anti-FRα IgE autoAbs were not detectable in patient serum (Supplementary Table). Although serum FRα and anti-FRα IgG autoAbs were measurable in 44% and 21% of patients, respectively (Figures 2D, 2F, Supplementary Table), basophils in 41 of 42 matched unfractionated blood samples were not activated by incubation with MOv18 or control IgE (Figures 2E, 2G). MOv18 IgE combined with monovalent recombinant FRα did not trigger activation (Supplementary Figure C). Moreover, no MOv18 IgE-mediated activation was measured in those 9% of patients with both measurable serum FRα and IgG autoAbs against FRα, or in the blood from 2 of the 3 patients who additionally had FRα-positive tumor (Figure 2H, Supplementary Table). Basophil activation by MOv18 IgE was observed in only one patient. In this patient's blood sample, we measured circulating FRα but no anti-FRα autoAbs. The patient's tumor FRα expression status was unknown and serum tryptase levels were not elevated (7 ng/ml; Supplementary Table). In the same patient, CD63 up-regulation was also triggered by the control non-FRα-reactive IgE. Together these suggested that basophil activation in this specimen may involve a non-FRα-specific mechanism, potentially through a humoral response directed towards the antibody's structural components. The prevalence of such a propensity to activate basophils in ovarian cancer and other patient cohorts and its potential clinical significance require further in-depth

93 investigations. Such studies may consider the possible cross-linking by autoAbs
94 such as those recognizing alpha-gal (galactose-α-1,3-galactose) previously
95 associated with hypersensitivity to cetuximab, an anti-EGFR IgG antibody[9], or by
96 anti-drug antibodies (ADAs) that may develop following MOv18 IgE treatment.
97
98 In conclusion, the basophil activation test showed no reactivity with MOv18 or
99 control IgE in 41 of 42 ovarian cancer patients' samples. Combined with
100 measurements of other clinical and biological parameters, application of BAT to
101 the clinical study of a first-in-class IgE in cancer patients (ClinicalTrials.gov
102 Identifier: NCT02546921) may allow correlations with clinical observations, to
103 help monitor and potentially predict patient safety.
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Figure Legends

Figure 1 – Anti-cancer IgE does not trigger basophil activation in 98% of cancer patient blood samples studied. Basophil activation (fold change in % CD63 expression) was evaluated following stimulation with anti-FcεRI antibody, anti-IgE antibody and fMLP (positive controls) and cross-linking of NIP IgE by multimeric NIP-BSA (A). No basophil activation (<3.0 fold change of % CD63-positive basophils, dotted cut-off line) was triggered by MOv18 or control IgE in all but one specimen, despite activation by positive controls (B-D), and irrespective of previous standard treatments received (E, F), nor when measured in the blood of a patient with already raised serum tryptase (G).

Figure 2 – FRα-positivity in blood or tumor does not influence basophil activation by anti-cancer IgE. Circulating FRα and anti-FRα autoantibodies may form immune complexes with MOv18 IgE, triggering basophil activation (A). No basophil activation was measured following MOv18 IgE stimulation in blood from the 71% of patients with FRα-positive tumor (B) (representative FRα-stained paraffin-embedded tumor, C). Despite detectable FRα, or anti-FRα IgG autoantibodies in a proportion of patients, MOv18 IgE triggered basophil activation in one blood sample (D-G). In the 9% of patients with both FRα and anti-FRα IgG autoantibodies, no basophil activation by MOv18 IgE or control IgE was observed (H).

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Ethical Approval

This study has been reviewed and approved by the Guy's Research Ethics Committee (Reference 09/H0804/45).

Conflicts of interest

SNK and JFS are founders and shareholders of IGEM Therapeutics Ltd., and HJB is now employed through a fund provided by IGEM Therapeutics Ltd. CB is a freelance pharmaceutical physician/medical advisor with Barton Oncology Ltd and in addition to work with Cancer Research UK Centre for Drug Development has undertaken consultancy work with many companies including in the last ~5 years, Astex Therapeutics Ltd, BerGen Bio A/S, Cancer Targeting Systems Inc, CellCentric Ltd, Certara LP, EngMab AG, Inbiomotion SL, Innate Pharma SA, Macrophage Pharma Ltd, , MorphoSys AG, Mosaic Biomedicals SL, Norgine Pharmaceuticals Ltd, Ono Pharma UK Ltd, Orion Clinical Services Ltd, Piqur Therapeutics AG, PTEN Research Foundation, SFL Services GmBH, Shionogi Ltd, T3 Pharmaceuticals AG, UCB Biopharma SPRL, and the Wellcome Trust Ltd. CB is on the advisory board for SFL Services GmBH and owns shares in GlaxoSmithKline. All other authors have declared no conflict of interest.

Author Contributions

HJB, DHJ and SNK conceived and designed the study. HJB, SK, CB, DHJ, and SNK helped with the development of the methodology. HJB, AK, CS, GP, CC, AR, KI, NW, CN-L, CG, SP, HJG, CJC, SJT, and DHJ acquired the data or helped with the data analysis and interpretation. AW, SG, AM, DHJ and JFS provided clinical support to HJB, AK, CS and CC to recruit patients. HJB, DHJ, JFS, and SNK discussed and interpreted the data and edited the manuscript. SNK supervised the study. HJB and SNK wrote the manuscript.

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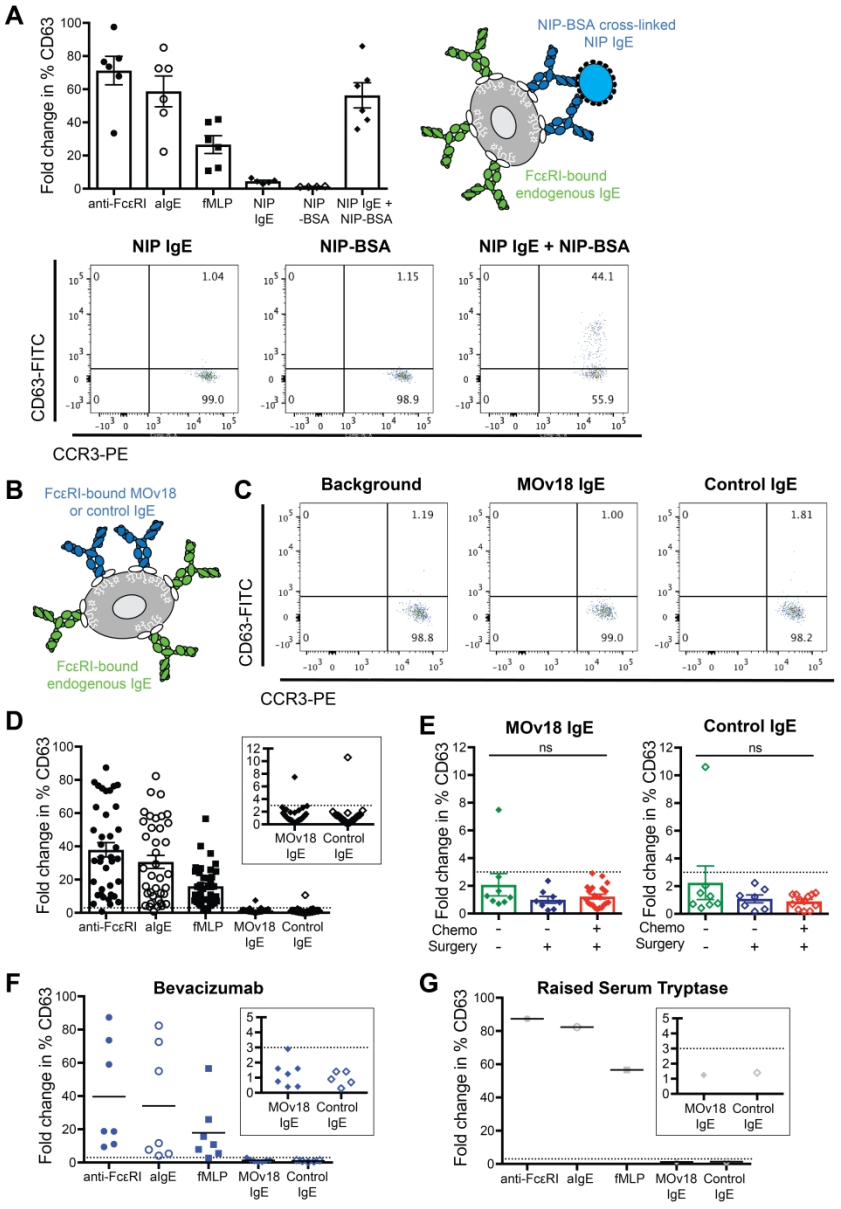
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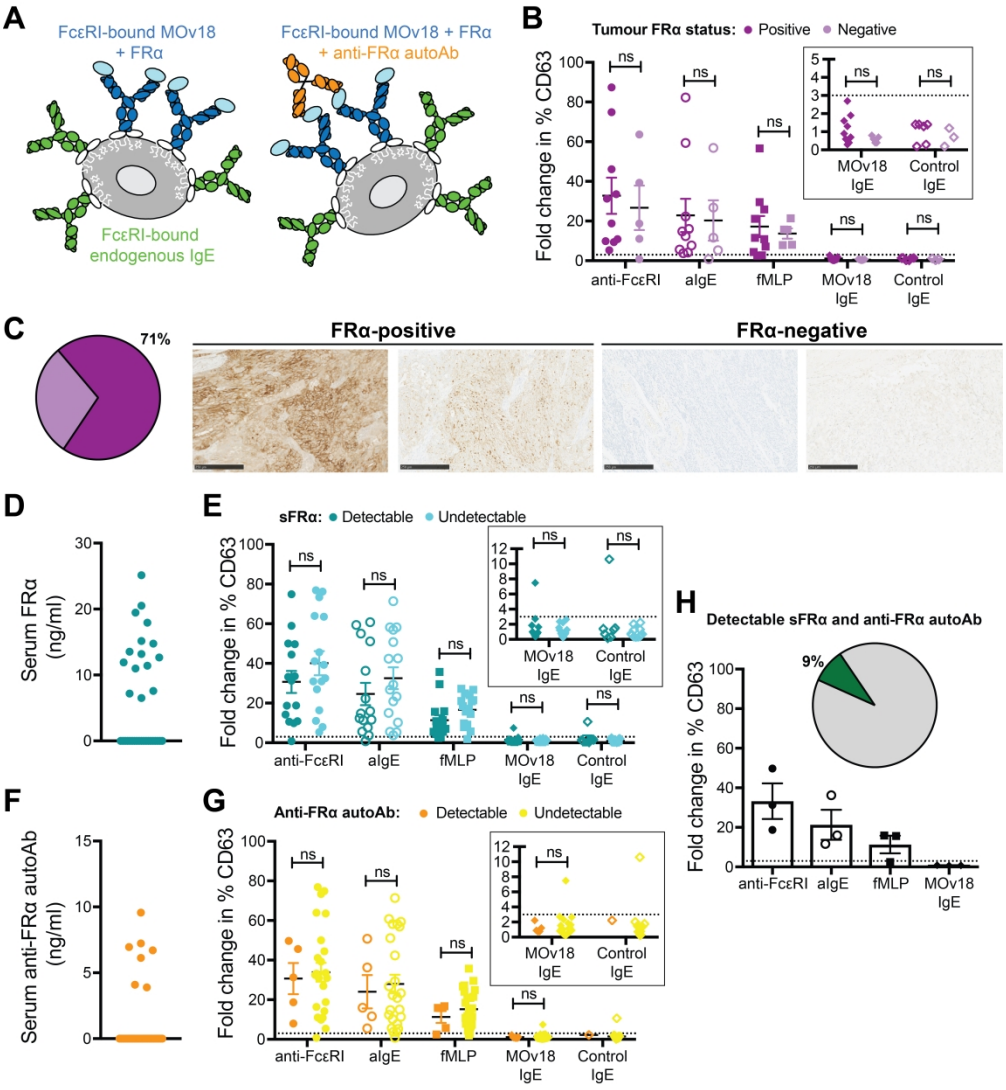
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Supplementary Methods

Ovarian cancer patient study

Women with ovarian cancer were enrolled into the study by written informed consent. Peripheral venous blood samples were drawn into BD Vacutainer™ Hemogard Closure Plastic K2-EDTA Tubes (BD). Serum samples were prepared by drawing blood into SST Clot Activator and Polymer Gel Hemogard Closure Blood Tubes (BD), followed by centrifugation of clotted blood at 2500RPM for 15 minutes at 4°C, careful pipetting of serum and storage at -80°C. Demographic characteristics, including tumor histology and prior treatment history, were obtained from clinical databases, anonymized and analyzed in conjunction with clinical samples.

Basophil Activation Test (BAT)

The basophil activation test (BAT) was performed within 4 hours of blood collection using the Flow2 CAST® kit (Bühlmann) as per instructions, except that incubation time with stimuli was optimized from the recommended 10 minutes to 30 minutes. Briefly, unfractionated whole blood was incubated with stimulation buffer (Bühlmann), and different stimuli: anti-FcεRI (Bühlmann), anti-IgE antibody (Dako) or fMLP (Bühlmann), or anti-FRα antibody, MOv18 IgE or control non-FRα-reactive IgE antibodies (at 3.5 µg/ml, prepared in-house). In the case of hapten-specific anti-NIP IgE, cross-linking with NIP-BSA (at 20 µg/ml, 5 NIP to BSA ratio, in-house) was included. Monovalent recombinant FRα (R&D Systems) was added, at indicated concentrations, to some MOv18 IgE stimulations. All conditions were then stained with anti-CCR3-PE and anti-CD63-FITC staining cocktail (Bühlmann) and incubated at 37°C for 30 minutes in a 5% CO_2 incubator. For all preparations, red blood cell lysis was then performed with diluted lysis buffer (Bühlmann) for 10 minutes at room temperature, followed by centrifugation and resuspension of cell pellets with acquisition buffer (Bühlmann). Flow cytometric evaluations were performed with a FACSCanto™ II using FACSDiva software (BD). Basophil activation was expressed as the fold change in % CD63-positive CCR3-PE^{high}SSC^{low} basophils over the background control (stimulation buffer and staining antibody cocktail alone) for each sample[1]. The % CD63 expression (without fold change calculation)

is also shown in Supplementary Figure B. All data analyses were performed, and representative plots prepared using FlowJo™ software (FlowJo LLC).

Tumor FRα expression status by immunohistochemistry (IHC)

For a subset of patients, tumor sections from primary debulking surgery were evaluated for FRα expression status. Novocastra™ Liquid mouse anti-human FRα primary antibody (Leica) was applied to formalin-fixed, paraffin-embedded tumor sections (from primary debulking surgery) for 32 minutes at room temperature at 1/500 dilution, followed by detection with Ultra Universal 3,3'-diaminobenzidine (DAB) detection kit (Ventana Medical Systems Inc.) and then Haematoxlyin II applied for 8 minutes. This protocol was performed using the BenchMark ULTRA automated immunohistochemistry/in situ hybridisation (IHC/ISH) slide staining system (Ventana Medical Systems Inc.), with an extended cell conditioning 2 (CC2) solution antigen retrieval.

Circulating FRα and anti-FRα autoantibody ELISAs

ELISAs were performed as previously described[2]. Circulating FRα (*e.g.* shed from tumor tissues) or anti-FRα autoantibodies in ovarian patient serum samples were evaluated by first coating 96-well MaxiSORP™ plates (Nunc) with 100 µl/well of 2 µg/ml monoclonal mouse anti-human FRα IgG1 antibody (clone 548908) or 1 µg/ml recombinant FRα, respectively (both R&D Systems and diluted in 0.2M carbonate-bicarbonate buffer, Pierce). Following incubation at 4°C, overnight, plates were blocked with 250 µl/well SuperBlock™ (Perbio Science Ltd.) for 2 hours at room temperature and then washed 4 times with 250 µl/well PBS-0.05% Tween® 20 solution (Severn Biotech and Sigma, respectively). Serum samples were diluted to 20% (or to 50% for IgE autoantibodies) in a 50:50 solution of SuperBlock™ and PBS-0.05% Tween® 20. Standard curves of recombinant FRα (R&D Systems), or anti-FRα human IgG or IgE monoclonal antibody (prepared in house) were diluted in SuperBlock™-PBS-0.05% Tween® 20, supplemented with 20% human serum albumin (type AB male, Sigma). Samples and standards were added 50 µl/well, in triplicate, and incubated for 2 hours at room temperature, followed by 4 washes. FRα was detected by 50 µl/well addition of biotinylated polyclonal goat anti-human FRα IgG1 antibody (R&D Systems, diluted to 25 ng/ml in SuperBlock™-

PBS-0.05% Tween® 20) for 2 hours at room temperature, 4 further washes, and 50 µl/well addition of streptavidin-peroxidase conjugate (Pierce, diluted 1/22000 in SuperBlock™-PBS-0.05% Tween® 20) for 30 minutes at room temperature. Anti-FRα IgG autoantibodies were detected by 50 µl/well addition of HRP-conjugated polyclonal goat anti-human Fcγ-specific $F(ab')_2$ fragment (Jackson Immuno Research, diluted 1/500 in SuperBlock™-PBS-0.05% Tween® 20) for 45 minutes at room temperature. Anti-FRα IgE autoantibodies were detected by 50 µl/well addition of HRP-conjugated polyclonal goat anti-human IgE antibody (Sigma, diluted 1/500 in SuperBlock™-PBS-0.05% Tween® 20) for 2 hours at room temperature. Plates were then washed 5 times and developed by 50 µl/well addition of OPD (Sigma) diluted to 0.5 mg/ml in stable peroxidase substrate buffer (Pierce) for 5-10 minutes, at room temperature, in darkness, followed by 50 µl/well 1M HCl solution (Sigma). Using a Fluostar Omega microplate reader (BMG LABTECH), FRα and anti-FRα IgG autoantibodies were measured using an absorbance 492nm, with a correction wavelength of 650nm. Standard curves were fitted using a 4-point variable curve-fitting program using a minimum of 6 points (MARS software, BMG LABTECH). The lower limit of quantification (LLOQ) was 6.25 ng/ml, 3.125 ng/ml, and 5 ng/ml for FRα, anti-FRα IgG, and anti-FRα IgE, respectively. Values below LLOQ are reported as 0 ng/ml.

Statistical Analyses

All statistical analyses were performed in GraphPad Prism (GraphPad Software, Inc.). Datasets were compared by t-test or one-way ANOVA with Kruskal-Wallis multiple comparisons. P values were represented as follows: *=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001. Error bars represent Standard Error of Mean (SEM).

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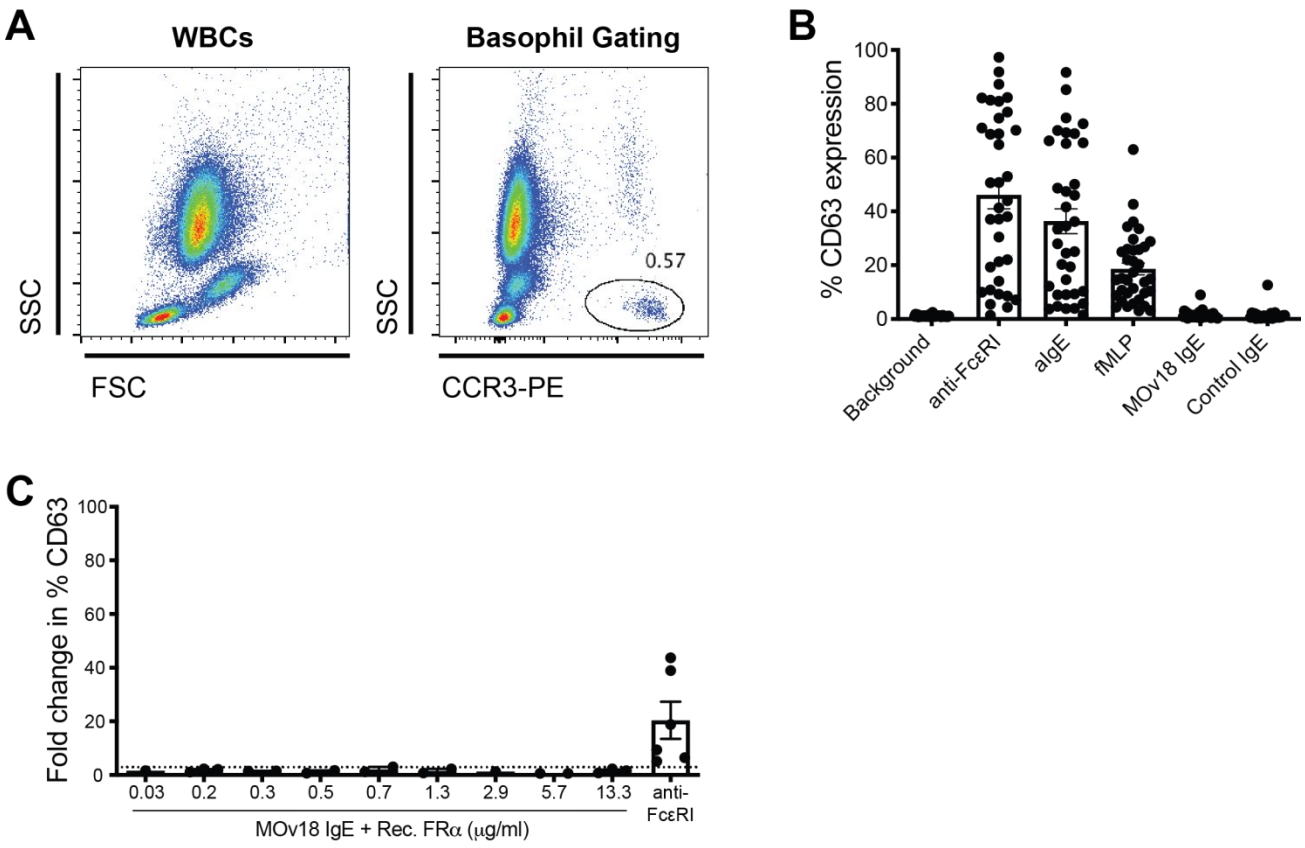

Supplementary Figure – (A) Basophil Activation Test (BAT) gating strategy. As per instructions for the Flow2 CAST® kit (Bühlmann), basophils in unfractionated whole blood samples were gated as $CCR3^{high}SSC^{low}$. Up-regulation of CD63 on the surface of basophils was monitored as a marker of *ex vivo* basophil activation; **(B) Basophil Activation Test (BAT) % CD63 expression without fold change calculation**; **(C) MOv18 IgE combined with recombinant FRα does not trigger basophil activation in patient blood.** No basophil CD63 up-regulation was measured following *ex vivo* stimulation with MOv18 IgE, plus monovalent recombinant FRα at concentrations up to 13.3 μg/ml, 500-fold higher than those measured physiologically in ovarian cancer patient circulation (highest FRα 25.13 ng/ml measured in our cohort (Figure 2D, Supplementary Table)).

Supplementary Table – Ovarian cancer patient characteristics.

	FRα status				BAT Fold change in %CD63	
	Tumor FRα expression	sFRα (ng/ml)	Anti-FRα IgG autoAb (ng/ml)	Anti-FRα IgE autoAb (ng/ml)	MOv18 IgE	Control non-FRα-reactive IgE
1	Positive	7.19	0.00	-	1.9	-
2	Negative	7.58	6.71	-	0.8	-
3	-	11.42	0.00	-	1.0	-
4	-	25.13	0.00	-	2.9	-
5	-	0.00	0.00	-	-	-
6	-	0.00	0.00	-	1.2	-
7	-	0.00	3.90	-	2.2	-
8†	Positive	0.00	9.57	-	-	-
9	-	19.47	0.00	0.00	7.5	10.6
10	-	11.90	4.10	0.00	0.9	-
11	Positive	13.19	0.00	0.00	0.6	-
12	Negative	0.00	0.00	0.00	0.6	-
13	Positive	20.52	6.13	0.00	0.7	-
14†	Positive	0.00	7.23	-	-	-
15	Negative	0.00	0.00	0.00	0.4	0.7
16†	-	0.00	0.00	0.00	-	-
17	-	0.00	6.96	0.00	1.2	2.2
18	-	0.00	0.00	0.00	2.4	1.8
19	Negative	6.51	0.00	0.00	0.4	0.2
20	Positive	0.00	0.00	0.00	0.3	0.2
21	-	0.00	0.00	0.00	0.2	0.2
22	-	0.00	0.00	0.00	1.5	1.3
23	Negative	0.00	0.00	0.00	0.7	1.2
24	-	0.00	0.00	0.00	1.3	2.0
25	-	0.00	0.00	0.00	2.6	0.8
26	-	0.00	0.00	0.00	0.8	0.4
27	-	0.00	0.00	0.00	0.6	0.3
28	-	0.00	0.00	0.00	1.2	0.7
29	Positive	14.75	0.00	0.00	0.9	1.3
30	-	-	-	-	0.8	0.6
31	-	12.67	0.00	-	1.6	1.5
32	-	15.16	0.00	-	0.7	0.7
33	Positive	13.54	0.00	-	-	-
34	-	-	-	-	0.8	1.2
35	-	-	-	-	0.3	0.6
36	Positive	-	-	-	1.6	1.4
37	Positive	17.95	0.00	-	0.4	0.3
38	Positive	10.99	0.00	-	2.7	1.4
39	-	-	-	-	1.8	1.0
40	-	-	-	-	1.6	0.9
41	-	-	-	-	1.9	1.5
42‡	Positive	-	-	-	1.3	1.4

†'Non-responder' patients, ‡Patient with elevated serum tryptase (33 ng/ml; ULN = 14 ng/ml). –Not tested.