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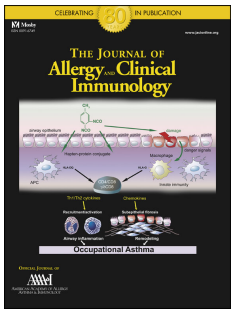

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CD23 surface density on B cells is associated with IgE levels and determines IgE-facilitated allergen uptake as well as activation of allergen-specific T cells

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ABBREVIATIONS

Art v 1: *Artemisa vulgaris* major allergen 1

AU: arbitrary units

Bet v 1: *Betula verrucosa* major allergen 1

CD23/ FcεRII: cluster of differentiation 23, low affinity IgE receptor

CD: cluster of differentiation (3, 11c, 14, 19, 27, 41a, 49d, 56, 61, 80, 86, 123)

CCR3: C-C chemokine receptor 3

EBV-B cells: Epstein-Barr virus transformed B cells

FcεRI: high affinity IgE receptor

FAB: facilitated antigen binding

FAP: facilitated antigen presentation

HLA-DR: human leucocyte antigen D related

Ig: immunoglobulin (E, D)

IL: interleukin (2, 4)

kU/L: kilo units per liter

MFI: mean fluorescence intensity

MHCII: major histocompatibility complex II

PBMCs: peripheral blood mononuclear cells

PMA: phorbol 12-myristate 13-acetate

PHA: phytohemagglutinin

R: R-value calculated with Pearson´s correlation

R_s: R-value calculated with Spearman rank correlation

sCD23: soluble CD23

SD: standard deviation

SPT: skin prick test

65 U/ml: units per milliliter

66 kUA/L: kilo units antigen per liter

67 kDa: kilo Dalton

68 FBS/FCS: fetal bovine serum / fetal calf serum

69

ABSTRACT

BACKGROUND: Increasing evidence suggests that the low-affinity receptor for IgE, CD23, plays an important role in controlling the activity of allergen-specific T cells via IgE-facilitated allergen presentation.

OBJECTIVE: To determine the number of CD23 molecules on immune cells in allergic patients and to investigate if the number of CD23 molecules on antigen-presenting cells is associated with IgE levels and influences allergen-uptake and allergen-specific T cell activation.

METHODS: Numbers of CD23 molecules on immune cells of allergic patients were quantified by flow-cytometry using Quantibrite beads and compared with total and allergen-specific IgE levels as well as with allergen-induced immediate skin reactivity. Allergen uptake and allergen-specific T cell activation in relation to CD23 surface density was determined by flow cytometry in combination with confocal microscopy and T cells transfected with the T cell receptor specific for birch pollen allergen, Bet v 1, respectively. Defined IgE-allergen immune complexes were formed with human monoclonal allergen-specific IgE and Bet v 1.

RESULTS: In allergic patients, the vast majority of CD23 molecules were expressed on naïve, IgD-positive B cells. The density of CD23 molecules on B cells but not the number of CD23-positive cells correlated with total IgE levels (R_S=0.53, p=0.03) and with allergen-induced skin reactions (R_S=0.63, p=0.008). Uptake of allergen-IgE complexes into B cells and activation of allergen-specific T cells depended on IgE binding to CD23 and was associated with CD23 surface density. Addition of monoclonal IgE to cultured PBMC significantly (p=0.04) increased CD23 expression on B cells.

CONCLUSION: CD23 surface density on B cells of allergic patients is correlated with allergen-specific IgE levels and determines allergen-uptake and subsequent activation of T cells.

CLINICAL IMPLICATION: Our study indicates a direct connection between IgE levels, CD23 surface density, allergen uptake and allergen-specific T cell activation. It may open new possibilities for controlling T cell-mediated allergic inflammation by targeting this pathway.

CAPSULE SUMMARY: Our study shows that the surface density of CD23 on B cells is associated with IgE levels and determines IgE-facilitated allergen uptake as well as activation of allergen-specific T cells in allergic patients.

INTRODUCTION

Immunoglobulin E (IgE) is known to have two different receptors, the high affinity receptor FcεRI and the low affinity receptor CD23 (FcεRII). Cross-linking of FcεRI-bound IgE by allergens mediates the degranulation of mast cells and basophils and thus leads to immediate symptoms of allergic disease[1]. In addition, FcεRI is expressed on eosinophils[2] and antigen presenting cells (APCs; e.g., monocytes, dendritic cells) and was shown to be involved in IgE-facilitated allergen presentation to T cells[3,4]. Interestingly, the expression of FcεRI on mast cells, basophils and even on antigen-presenting cells is up-regulated by increasing IgE levels[5,6] and it was found that omalizumab, an anti-IgE antibody, prevents IgE binding to FcεRI and thereby also down-regulates FcεRI expression[7].

The expression of the low affinity receptor for IgE (CD23), a 45 kDa calcium-binding protein belonging to the family of C-type lectins on various cell types has mainly been investigated using cells cultured under various conditions. These studies have shown that CD23 is expressed on B cells[8], monocytes[9], T cells[10], dendritic cells[11] platelets[12] and neutrophils[13]. However, the expression of the numbers of CD23 molecules on these cell types has not been studied in detail using *ex vivo* isolated cells from allergic patients. CD23 has an important function in IgE-facilitated allergen presentation to T cells[14, 15]. In fact, IgE-facilitated antigen presentation (FAP) strongly activates allergen-specific T cells and the secretion of pro-inflammatory and Th2-driving cytokines[14-17]. It has been shown that FAP can be inhibited with a therapeutic anti-CD23 antibody[18] and by allergen-specific IgG antibodies induced by allergen-specific immunotherapy[19]. An association between improvement of symptoms after specific immunotherapy with a reduction of allergen-IgE binding to CD23 (facilitated antigen binding, FAB) on B cells by enhanced levels of blocking IgG antibodies has been demonstrated using FAB assays[20,21].

Despite the importance of CD23 in activating allergen-specific T cells, several aspects of its biology have not been investigated as meticulously as for FcεRI. For example, there are no studies which have investigated the density of the expression of CD23 molecules on *ex vivo* isolated cells from allergic patients. Studies investigating CD23 mainly focused on the relative number and percentage of cells expressing CD23[22-29]. Therefore it has also not been studied if the number of CD23 molecules on the cells is associated with total and allergen-specific IgE levels. Furthermore, there are no systematic studies in defined experimental human model systems which have analysed if and how the number of CD23 molecules on antigen-presenting cells has an effect on the magnitude of IgE-facilitated allergen presentation and subsequent T cell activation.

In the present study we established a new technique for the measurement of the precise CD23 receptor molecule numbers on the surface of immune cells in order to investigate the distribution frequency of CD23 on immune cells in allergic patients and if and how this parameter is correlated with IgE levels. We also studied whether addition of IgE to PBMC cultures has effects on CD23 expression on B cells. Furthermore, we used CD23 cell lines expressing different numbers of CD23 molecules on their surface to study if and how the density of CD23 molecules on APCs influences IgE-facilitated allergen uptake and allergen-specific T cell activation.

METHODS

Patients

Blood samples from seventeen study participants with a positive history suggestive of grass pollen allergy and a positive skin prick test reaction with grass pollen extract were analysed. Apart from their allergy, none of the subjects had a history of a chronic or current acute disease. Subjects were included in the study during the grass pollen season (i.e., during the months of June/July in Vienna). The presence of symptoms of grass pollen allergy (rhinitis, conjunctivitis, asthma) was recorded at that time. Furthermore, a history of other allergies was obtained. No patients were analysed who had a contraindication against skin prick testing or were under long-term treatment with systemic corticosteroids, immunosuppressive drugs, tranquilizers or psychoactive drugs. Prior to the study, patients were not allowed to use oral antihistamines for 3 days and local (in the skin test area) and systemic corticosteroids for 14 days. The blood samples were analysed in an anonymized manner with approval of the Ethics Committee of the Medical University of Vienna (EK508/2011) after written informed consent was obtained from the patients.

Skin prick tests

Skin test solutions (Timothy grass pollen extract, positive control: codeine phosphate, negative control solution, Stallergenes, France) were applied to the lower arms of patients and were pricked with commercial prick lancets (Allergopharma, Reinbek, Germany). After 20 minutes, the wheal reaction was surrounded with a felt pen and transferred to paper using adhesive tape. The size of the wheal reactions was measured by planimetry as described.[30]

Blood samples, total and allergen-specific IgE measurements

Immediately after venepuncture from the antecubital vein the cells from heparinized blood samples were assessed for CD23-expression. Serum was obtained from clotted blood samples by centrifugation and was stored at -20℃ until use. Total IgE and timothy grass pollen-specific IgE levels were measured using the Phadia CAP system (Thermofisher, Uppsala, Sweden).

Analysis of the data

All clinical data (patient history, skin prick test results) were obtained by a clinical investigator and deposited in a data base. Measurement of total and specific IgE levels was performed by an independent external laboratory, which was unaware of clinical data and CD23 measurements. Measurement of CD23 levels on different cell types was performed by another independent investigator who was not in contact with the study participants and who was blinded regarding total and specific IgE levels and regarding clinical data (i.e., results from skin prick tests and clinical symptoms). After all three independent data sets (i.e., IgE levels, clinical data, CD23 measurements) were completed they were submitted to a data base and correlations were analysed.

Blood sample preparation and flow cytometry

Red blood cell lysis solution (155 mM ammonium chloride, 10 mM potassium bicarbonate, 12 mM EDTA) was applied to heparinized patients' blood samples. For flow cytometry, the following surface markers of cells were stained: T cells (positive with anti-CD3, clone OKT3), NK cells (anti-CD3 negative, positive with anti-CD56, clone TULY56), B cells (positive with anti-CD19, clone HIB19), monocytes (positive with anti-CD14, clone 61D3), platelets (positive with anti-CD61, clone VI-PL2 and

with anti-CD41a, clone HIP8), basophils (positive with anti-CD123, clone 6H6 and anti-CCR3, clone 5E8-G9-B4), neutrophils (granulocytes negative with anti-CD49d, clone HP2/1), eosinophils (granulocytes positive with anti-CD49d, clone HP2/1, anti-CD19 negative), dendritic cells (lineage cocktail negative, positive with anti-CD11c, clone 3.9), naïve B cells (positive with anti-CD19, clone J3-119 and anti-IgD, clone 11-26), memory B cells (positive with anti-CD19, clone J3-119, positive with anti-CD27, clone O323). All cells were additionally stained with anti-CD23 (clone EBVCS2). Matching non-binding isotype antibodies were used as controls. All antibodies were obtained from eBioscience (San Diego, CA, USA) except for anti-CD49d, anti-CD19 for naïve/memory staining (Beckman Coulter, Brea, CA, USA) and lineage cocktail lin1 (BD biosciences, Franklin Lakes, NJ, USA). Aliquots of $1.5x10^6$ cells were used for each staining. Before staining, cells were blocked with 10% v/v mouse serum (Life Technologies, Carlsbad, CA, USA). Dead cells were excluded from the analysis with e780 fixable viability dye (eBioscience). Flow cytometry analysis was performed on a Beckman Coulter FC 500 flow cytometer (Beckman Coulter, CA, USA). Depending on the cell type, $3x10^5$ (T cells, B cells, NK cells, monocytes), $1x10^6$ (Basophils), or $5x10^5$ (all other cell types, i.e., neutrophils, eosinophils, dendritic cells, platelets) events were recorded. FlowJo Software 7.5 (Treestar Inc., Ashland, OR, USA) was used for data analysis. Gates were set according to the matching non-binding isotype control of each antibody for each cell type.

Measurement and calculation of CD23 surface density

Quantification of CD23 expression was performed using BD Quantibrite PE beads (BD biosciences) according to the manufacturer's instructions. Briefly, beads with different intensity levels in PE-channel FL-2 and defined numbers of surface PE

molecules were used as a standard in flow cytometry and used for back-calculation of CD23 stained with a PE-labelled anti-CD23 antibody. FlowJo Software 7.5 (Treestar Inc., Ashland, OR, USA) was used for data analysis. Molecule density on cells was calculated only when more than 20 cells of the assessed cell type were positive for CD23.

Measurement of soluble CD23 in sera

Measurement of sCD23 in the sera of the patients was performed with Novex® CD23 (soluble) Human Direct ELISA Kit (Life Technologies) according to the manufacturer's instructions. The ng/ml values of soluble CD23 were calculated with a standard measuring the 25 kDa form of sCD23.

Recombinant allergens, uptake of IgE-allergen complexes by CD23-expressing B cell lines

Recombinant Bet v 1 and Art v 1 were obtained from Biomay AG (Vienna, Austria). A recombinant trimer of Bet v 1[31] was used for experiments where high fluorescence intensity was required. Three EBV-transformed, CD23-expressing B cell lines (high, medium and low CD23 expression) were generated as previously described[32]. For each sample of 1-2 x 10^5 EBV transformed B cells 300 ng of a Bet v 1-specific recombinant monoclonal antibody with a human IgE heavy chain constant region[33] were incubated with 300 ng of Bet v 1 trimer fluorescently labelled with Dylight-488 according to the manufacturer's instructions (Pierce DyLight 488 labelling kit, Thermo Scientific, Rockford, USA). Bet v 1 trimer[31] was fluorescently labelled with pHrodo™ Green STP Ester (Life Technologies) according to the manufacturer's instructions. The cells were first blocked with 10% human serum for 20 minutes at 4℃. Subsequently, the IgE-allergen complex or allergen alone was added to the cells and

incubated for 20 minutes at 4℃. The cells were washed with PBS and incubated for 3.5 or 5 hours in RPMI medium (Gibco, Life Technologies) at 37℃, 5% CO_2. For Dylight-488 labelling, surface fluorescence was removed by acid wash immediately after the 20 minutes incubation at 4℃ as well as after 3.5 and 5 hours incubation at 37℃. For this purpose cells were exposed to acid wash I (130 mM NaCl, 0.5 mM KCl, 10 mM lactic acid, pH 3.9)[34] three times for 5 minutes and additionally to acid wash II (150 mM NaCl, 20 mM HCl, pH 1.7)[35] for two times one minute at room temperature. Surface CD23 was stained by anti-CD23-PE antibody or matching isotype control. Binding of fluorescently labelled allergen binding (0h) or uptake (3.5h, 5h) by the cells were measured by flow cytometry as stated above.

CD23 dependency of binding and uptake of IgE-allergen complexes was shown with an anti-CD23 antibody (clone M-L233, BD Biosciences). Anti-CD23 or a matching isotype control (mouse IgG_1) was incubated with the cells at a concentration of 15 µg/ml for 20 minutes on ice before incubation with the allergen-IgE complexes.

Uptake of fluorescently (Dylight 488)-labelled allergen in confocal microscopy was visualized using a Zeiss LSM 510 (Zeiss, Oberkochen, Germany) with an oil immersion 60x lense. For this purpose, the silhouettes of cells were stained with anti-CD19 (alexa fluor 647, clone HIB19, Biolegend, San Diego, USA) after incubation with allergen-IgE complexes and after acid wash. Cells were then added to adhesion slides (Paul Marienfeld, Lauda-Koenigshofen, Germany), fixed with acetone, washed twice with PBS and the slides were covered with Fluormount (Sigma-Aldrich, St. Louis, USA). A representative cell sample for each cell line was chosen for depiction.

Cell sorting and T cell activation assay

An EBV-immortalized B-cell line (HLA-DRB1:0701) was cytometrically sorted for CD23 expression (FACS Aria, Becton Dickinson). Briefly, EBV-immortalized B-cells

cells were stained with α-human CD23-FITC (TU1, Caltag, Invitrogen-Fisher Scientific) for 30 min at 4℃, washed once with 1xP BS supplemented with 0.5% w/v BSA and incubated with α-human CD11a (Efalizumab, $3x10^6$ EBV cells, 5-10 µg/ml) for 30 min at 4℃ to prevent homotypic aggregation of the EBV-immortalized B-cells[36]. Cells were washed once with 1xPBS/0.5% w/v BSA and subsequently sorted for CD23 high (MFI: 325), intermediate (MFI: 138) and negative/low (MFI: 11) expressing EBV-immortalized B-cells (FACS Aria).

After 48 hours of recovery, sorted EBV-immortalized B-cells ($5x10^4$/well) were incubated with titrated concentrations of allergen/anti-Bet v 1 specific IgE complexes in V-bottom plates at 37℃ for 3 h. The dependency of IgE binding on binding to CD23 was investigated by adding an anti-CD23 antibody (clone M-L233, BD Biosciences) or a matching isotype control (mouse IgG_1) to the cells. After washing with 1xIMDM $1x10^5$ Bet v $1_{142–156}$-specific Jurkat T cells[37] stably transfected with an IL-2-luciferase reporter were added to each well and co-cultured for 6 h. PMA (10^{-7} M) plus PHA (12.5 µg/ml) served as positive and medium alone as negative control, respectively. After the co-incubation period, cells were lysed and luciferase activity was determined (Promega, Madison, WI) on a Luminoskan Ascent luminometer (Thermo Scientific) as described.[38]

Flow cytometry measurement of MHC and co-stimulatory molecules

Immunophenotyping of sorted EBV-immortalized B-cells was performed with directly conjugated monoclonal antibodies directed against HLA class II (HLA-DR, L243, FITC), CD80 (2D10, PE), CD86 (IT2.2, APC) and CD19 (HIB19, PerCP) as well as non-binding isotype control antibodies (Biolegend). Four-color data acquisition was performed on a fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson) and analysed by the CellQuest software (Becton Dickinson). Mean

fluorescence intensity (MFI) data were calculated from the geometric mean of the fluorescent intensity for all positive cells (above isotype control) in the flow cytometry channel used. Only data acquired in the same experiment with the exact same settings were used to compare MFIs.

Stimulation of PBMCs with IgE

PBMCs from blood donors (5 allergic patients with total IgE levels between 200-312 kU/L; 3 non-allergic subjects with total IgE levels <100 kU/L) were isolated by Ficoll gradient centrifugation (GE Healthcare). Aliquots of $1x10^6$ cells/well were cultivated in 1x RPMI medium (Life Technologies) in 12-well plates together with 1 µg/ml of a purified recombinant monoclonal human IgE antibody[33] or with PBS alone (Life Technologies). All experiments were done in duplicates. CD23 expression on B cells was assessed by flow cytometry after 6 days of culture. To this end, $5x10^5$ total cells were assessed and B cells were stained with an anti-CD19 antibody (clone HIB19) and with an anti-CD23 antibody (clone EBVCS2). Quantification with Quantibrite beads was done as described above and results represent means of duplicates with an error of <5%.

Statistical Analysis

To calculate correlations, we used Pearson's correlation coefficient (R) or Spearman's rank correlation coefficient (R_s), depending on the distribution of the data. Spearman's rank correlation coefficient (R_s) was therefore used for correlation of patient's CD23 with IgE and skin sensitivity values because of the skewed distribution of the data. For correlation in *in vitro* B cell uptake and T cell activation experiments, Pearson's correlation coefficient (R) was used because of the normal distribution of the data. Coefficients were considered significant at a p-value < 0.05.

A paired t-test was performed to test the null hypothesis, stating that the mean relative increase of CD23 expression after addition of IgE to PBMCs is zero.

The analyses were performed with the software package R version 3.1.1. Significant p-values below 0.05 (two-sided test) are marked with * in figures. P-values below 0.01 (two-sided tests) are marked with **.

RESULTS

Characterization of grass pollen allergic patients

Blood samples from seventeen patients (9 males, 8 females) with a history of grass pollen allergy (i.e. rhinitis, conjunctivitis and/or asthma during the grass pollen season May-July) were analysed. Clinical and demographic data of the patients are displayed in Table I. All patients had current symptoms of grass pollen allergy (Table I) when skin prick tests were performed and blood samples for flow cytometry and other experiments were obtained. All but two patients had a history of other allergies. Total IgE levels ranged between 33 and 10856 (median: 462) kU/L while IgE levels specific for timothy grass pollen ranged between 1.1 and 1204 (median: 33) kUA/L. All patients had positive skin prick test reactions to grass pollen extract (12.6 -238.7 mm^2; median: 61)

In the grass pollen allergic patients CD23 was expressed mainly on naïve IgD-positive B cells.

When CD23 expression was determined on blood cells from patients who were suffering from symptoms of grass pollen allergy, we found that the receptor was expressed almost exclusively on B cells. Table IIA shows that in average 49.8% of patients' B cells expressed CD23 molecules, whereas in most patients only few monocytes (mean: 1.38%), NK cells (mean: 0.57%) or dendritic cells (mean: 0.43%)

were CD23 positive (Table IIA). Next, we investigated the CD23 receptor density on the CD23 positive cell populations (Table IIB). We found a high density of CD23 molecules expressed on B cells (2168-6719 molecules/cell; mean: 3968) whereas in only 6 out of the 17 patients it was possible to calculate the number of CD23 molecules on monocytes and the density was lower than on B cells (i.e., 388-1465 molecules/cell; mean: 1089/cell).

In order to characterize the CD23 positive and CD23 negative populations of B cells, we stained blood cells of 3 patients (#8, #11, #17) with antibodies specific for CD19, CD23, IgD and CD27 (Table E1). On an average, 65.1% of B cells were CD23 positive in these patients. The majority of the CD23 positive cells were also positive for IgD and negative for the memory marker CD27 (naïve B cells, mean: 89.2%) (Table E1). Only 0.5% of the CD23 positive B cells were IgD/CD27 double positive pre-switch memory B cells, 1.1 % were CD27 positive, IgD negative post-switch memory cells and 9.2 % were IgD/CD27 double negative resting memory B cells.

Within the CD23 negative B cell population (i.e., 34.9 % of all B cells), 42.9% were naïve IgD positive, CD27 negative B cells, 47.1% were IgD/CD27 double negative resting memory B cells, 8.9% were CD27 positive, IgD negative post-switch memory cells and 1.1% of the CD23 negative B cells were pre-switch memory B cells (IgD/CD27 double positive) (Table E1). The flow cytometry charts of one representative patient (#11; Table I) are displayed in Fig E1 in the online repository.

Total IgE levels are correlated with the number of CD23 molecules on B cells

In our group of 17 grass pollen allergic patients, we found a significant correlation between the density of CD23 on CD23 positive B cells (Table IIB) and total serum IgE levels (R_S=0.53, p=0.03, Fig 1A). A pilot experiment showed that addition of purified monoclonal human IgE to cultured PBMC resulted in a significant increase

(mean: 8.5%; p=0.04) of CD23 expression on B cells suggesting that IgE may up-regulate CD23 expression (Fig E2, online repository).

Furthermore, grass pollen-specific serum IgE levels were also linked to the number of CD23 molecules on B cells (R_S =0.46, p=0.07, Fig 1B). We also found a significant correlation between the molecule density of CD23 on B cells (Table IIB) with the size of skin prick test reactions to grass pollen extract (R_S=0.63, p=0.008, Fig 1C). Additionally, size of skin prick test reactions correlated significantly with grass pollen-specific serum IgE levels (R_S=0.54, p=0.03) (data not shown). Since only 6 patients showed substantial CD23 expression on monocytes we did not correlate the number of CD23 molecules on monocytes with respective IgE and skin prick test data.

Neither the numbers of CD23 positive B cells nor soluble CD23 levels are correlated with total and specific IgE levels or skin sensitivity.

Figure 1 shows also the correlations of the percentage of CD23 positive B cells with total and allergen-specific IgE as well as with grass pollen-specific wheal reactions. In fact, neither total serum IgE levels (R_S=0.34, p=0.19, Fig 1D) nor grass pollen specific serum IgE levels (R_S=0.34, p=0.19, Fig 1E) correlated with the number of CD23 positive B cells. We also did not find a statistically significant correlation between the percentage of CD23 positive B cells with skin prick test reactivity to grass pollen (R_S=0.42, p=0.09, Fig 1F).

We analysed also the levels of soluble CD23 in the sera of the 17 patients (Table IIB). However, soluble CD23 serum levels did not correlate with patient's total IgE (R_S=-0.12, p=0.66), grass pollen-specific IgE (R_S=-0.33, p=0.19) or grass pollen-specific wheal reactions (R_S=-0.06, p=0.81) (data not shown).

IgE-facilitated allergen uptake is dependent on CD23 density on B lymphocytes

We used 3 different CD23-expressing EBV-transformed B cell clones in order to investigate the possible dependence of IgE-facilitated allergen uptake into B cells on CD23 density. Cell line I had an average number of $3x10^5$ CD23 molecules/cell, cell line II expressed $1.2x10^5$ CD23 molecules/cell on an average and cell line III $5x10^4$ molecules/cell on an average (Fig 2). In a first set of experiments, we measured and compared the uptake of the complexes into the different cell lines after removal of bound surface molecules via acid wash by incubating the cells with fluorescently labelled allergen-IgE complexes. The allergen-uptake into the different cell lines was studied by confocal microscopy and CD19 positive cells were stained (Fig 2A). We found that the staining of allergen-IgE immune complexes was more intensive on the cell lines expressing high levels of CD23 molecules (i.e., cell line I and II) as compared to the cell line expressing fewer CD23 molecules (i.e., cell line III) (Fig 2A). Cells had been stripped with an acidic wash after incubation with the fluorescently-labelled IgE-allergen complexes followed by anti-CD19 staining. Under these conditions CD19 stained the silhouette of the sections whereas labelled allergen appeared intracellular indicating that the allergen had been taken up. No allergen-staining was observed with any of the three CD23-expressing cell lines when allergen-specific IgE was omitted (data not shown).

The findings of the confocal staining experiments were corroborated by a second set of experiments in which allergen-IgE binding to the cell surface (Fig 2B) and uptake into the cells (Fig 2C) were studied by flow cytometry. In these experiments we found that there was a significant correlation ($R=0.7$; $p=0.01$) between the number of CD23 molecules on the cells and the binding of the allergen-IgE complexes after 20 minutes incubation at 4℃ (Fig 2B). An almost linea r and striking correlation ($R=0.98$; $p=0.000$; Fig 2C) was found between the uptake of allergen after 3.5 hours of incubation at 37℃ and the surface density of CD23 molecules on these cells.

Binding as well as uptake of allergen-IgE complexes could be blocked by an anti-CD23 antibody (Fig 2D, 2E), and both, complex binding and uptake were unaffected if a matching isotype control was used instead (Fig 2D, 2E). Our experiments demonstrate the causal dependency of allergen uptake and T cell activation on IgE-facilitated allergen presentation via CD23. In these experiments the extent of IgE-facilitated allergen uptake was correlated with the surface density of CD23.

In order to further confirm that the allergen-specific signals measured via flow cytometry were derived only from intracellular compartments and did not represent remaining allergen residing on the cell surface, we repeated the uptake experiment using allergen labelled with the pH-sensitive pHrodo green which only emits fluorescence after uptake into endo-lysosomal compartments due to acidic pH. Again, the measured uptake of allergen significantly correlated with the CD23 surface density (R=0.97, p=0.000, data not shown).

The density of CD23 on the surface of B cells determines the activation of allergen-specific T cells by IgE-facilitated allergen presentation

Next, we measured if the density of CD23 on antigen-presenting B cells which had been loaded with IgE-allergen complexes influences the activation of MHC-matched Bet v 1-specific T cells which expressed a single Bet v 1-specific T cell receptor. For this purpose, we sorted EBV-transformed B cells into three different sub-populations according to their CD23 expression levels (high, intermediate or low expression), incubated them with equal amounts of Bet v 1-IgE complexes and measured Bet v 1-specific T cell activation (Fig 3). Our experiments showed that T cell activation was directly dependent on the CD23 surface density of co-cultured B cells when incubated together with allergen-IgE complexes (Fig 3A and B). Initial CD23 expression on B cells was correlated with subsequent T cell activation (R=0.94;

p=0.000, Fig. 3A). No relevant T cell activation was observed when an unrelated allergen (Art v 1) or Bet v 1 without Bet v 1-specific IgE was used for stimulation (Fig 3B) and IgE-facilitated T cell activation could be blocked by an anti-CD23 antibody (Fig 3C). Additionally to CD23, we also measured MHCII, CD80 and CD86 expression on the sorted B cells. We found some differences in the expression of co-stimulatory molecules CD80 and CD86. However, there was no difference regarding MHC class II expression in CD23 high and CD23 intermediate cell populations although T cell activation was different (Table E2).

DISCUSSION

Several studies performed in patients during allergen-specific immunotherapy (SIT) have provided evidence that CD23, the low affinity receptor for IgE, has an important role in controlling allergen-specific T cell activation via IgE-facilitated allergen presentation[19,20,21,39]. In order to investigate the number of CD23 molecules on immune cells of allergic patients we have developed a flow cytometry-based assay using Quantibrite beads. Using this assay we found that CD23 is expressed primarily on naïve IgD-positive B cells in the blood of allergic patients and only in a negligible manner on other immune cells.

In fact, CD23 was present on other antigen presenting cells such as dendritic cells or monocytes, however, only few of these cells were CD23 positive and/or expression levels were very low (Table 2A). Therefore it was not possible to calculate reliable numbers of CD23 on their cell surfaces. We therefore must assume that mainly B cells play a role in IgE-facilitated allergen presentation via CD23. Interestingly, the number of CD23 molecules per cell and not the percentage of CD23-positive cells was correlated with total IgE levels and allergen-induced immediate type skin reactions and was associated with allergen-specific IgE levels. Pilot experiments even suggested that addition of highly purified human monoclonal IgE may up-regulate CD23 expression on B cell in cultured PBMC from allergic and non-allergic subjects. The surface expression of CD23 thus seems to follow the rules which guide the expression of the high affinity receptor for IgE, FcεRI, which was shown to depend on IgE levels[5] and to be regulated directly by IgE binding[40]. In fact, it has also been shown that an anti-IgE antibody (omalizumab) which prevents binding of IgE to FcεRI by shielding IgE binding to the receptor can down-regulate FcεRI expression on basophils, mast cells and antigen presenting cells[7].

Next, we were interested if the surface density of CD23 on B cells may have an influence on IgE-facilitated allergen uptake and subsequent allergen-specific T cell proliferation. To study this question we employed defined model systems. We used the purified major allergen of birch, Bet v 1, a human monoclonal Bet v 1-specific IgE antibody, T cells transfected with a Bet v 1-specific T cell receptor and MHC-matched, EBV-transformed B cells which were characterized regarding the numbers of CD23 molecules on their surface. The experiments performed showed that allergen-uptake by IgE-facilitated allergen presentation was significantly correlated with the numbers of CD23 molecules on the surface of the APCs and depended on the presence of allergen-specific IgE. It was causally related to the ability of IgE to bind to CD23 because it could be blocked with an anti-CD23 antibody. Furthermore, strongest T cell activation was observed when APCs expressing high levels of CD23 were used for IgE-facilitated allergen presentation and we found a significant correlation ($p=0.000$) between CD23 levels on B cells and T cell activation. Taken together, our findings suggest that high levels of allergen-specific IgE up-regulate the surface density expression of CD23 on B cells in allergic patients which then may lead to enhanced IgE-facilitated allergen-presentation and activation of allergen-specific T cells. This possible interplay between IgE levels, CD23 surface density on B cells as APCs and allergen-specific T cell activation may open possibilities to control T cell-mediated allergic inflammation due to IgE-facilitated allergen presentation by several, non-directly T cell- or cytokine-targeting approaches and non-pharmacological strategies. For example, it may be considered to block the CD23-binding site on IgE with anti-IgE antibodies as has been suggested earlier[41,42] to suppress allergen-specific T cell activation. In this context, it was found that omalizumab indeed suppressed markers of T cell activation when administered to patients suffering from atopic dermatitis[43]. Another possibility would be to block

binding of IgE to CD23, for example with therapeutic anti-CD23 antibodies or CD23 variants[44]. In this context, it was found that lumiliximab, an anti-CD23 antibody suppressed allergen-specific T cell activation *in vitro*[18]. Also other "humoral" approaches which primarily focus on IgE production[45] and the interference of the IgE-allergen interaction (e.g., passive immunization with blocking allergen-specific IgG[46]) may have an influence on T cell-mediated allergic inflammation.

In summary, our study suggests that IgE may play a role in the upregulation of CD23 on B cells and demonstrates that the surface density of CD23 on B cells determines allergen-uptake and subsequent T cell activation. These findings conceivably have implications for the treatment of allergic diseases.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

TABLE I. Demographic and clinical data of grass pollen allergic patients. Shown are sex, age, current symptoms to grass pollen, sensitization to other allergen sources, total IgE levels (kU/L), timothy grass pollen-specific IgE levels (kUA/L) and sizes of wheal reactions to grass pollen mix (mm^2). SPT: skin prick test.

TABLE II. A, Percentages of CD23-positive cells among different cell types. CD23 positive cells in blood samples from the grass pollen allergic patients (Table I; n=17). Shown are the mean percentages and the standard deviation (in parentheses, SD) of CD23 positive cells within the investigated cell types and the ranges. **B,** Numbers of CD23 molecules per cell and concentrations of soluble CD23 in serum. The average numbers of CD23 molecules per CD23-positive B cell and monocyte and the concentrations of soluble CD23 in patient's sera (ng/ml) were measured in the blood of the 17 grass pollen allergic patients (Table I). n.d.: Numbers of CD23 molecules could not be calculated when less than 20 of the monocytes among the analyzed cells were positive for CD23.

FIG 1. Associations of the numbers of CD23 molecules per B cell and of the percentage of CD23 positive B cells with skin sensitivity to grass pollen allergens and total and grass pollen-specific IgE levels. Numbers of CD23 molecules per CD23-positive B cell (**A-C**) or percentages of CD23-positive B cells (**D-F**) are displayed on the y-axis. Total IgE levels (**A, D**), timothy grass pollen-specific IgE levels (**B, E**) or sizes of grass pollen-induced wheal reactions (**C, F**) are plotted on the x-axis. P-values < 0.05 are marked with * and p-values below < 0.01 with **.

FIG 2. Dependence of allergen-uptake on the CD23 cell surface density. **A,** Confocal microscopy of three EBV-transformed B cell lines expressing different numbers of CD23 molecules on their surface (I: $3.5x10^5$, II: $1x10^5$, III: $5x10^4$ CD23 molecules / cell). Cell were stained with anti-CD19 in pink whereas allergen (Bet v 1 Trimer) was labeled in green with dye 488, pre-incubated with allergen-specific IgE and then exposed to the cells. The pictures marked "overlay" show an overlay of the anti-CD19 and allergen staining. **B,** Binding and **C,** uptake of a fluorescent allergen-IgE complex by CD23-expressing EBV-transformed B cell lines (I, II, III) shown by flow cytometry. CD23 expression was measured before and after 3.5 hours of incubation with IgE-allergen complexes. Different symbols (I: circles; II: squares; III: triangles) represent the three different B cell lines. Four independent experiments performed on four different study days are displayed. Each data point represents the mean of triplicate experiments which were done on the same study day. Background fluorescence measured after incubation with allergen alone was subtracted. Dotted lines: trend lines of the correlations. **D,** Binding and **E,** uptake of allergen-IgE complexes was blocked by an anti-CD23 antibody, but not by the matching isotype control (IgG_1). Closed triangles represent blocking by anti-CD23, open circles show cells incubated with isotype control.

FIG 3. IgE-facilitated T cell activation is dependent on the CD23 surface density. EBV-immortalized B cells were sorted according to their CD23 expression (white: low expression, grey: intermediate expression, black: high expression) **A,** Correlation between the numbers of CD23 molecules on B cells (x-axis) and T cell activation (y – axis: luciferase activity in arbitrary units) induced by IgE-Bet v 1 immune complexes. Four independent experiments on four different study days are displayed. Dotted lines: trend lines of the correlation. **B,** Activation of Bet v 1-specific T cells (y-axis)

after incubation of sorted EBV-immortalized B cells with IgE-Bet v 1 immune complexes, Bet v 1-specific IgE and the unrelated allergen Art v 1, or Bet v 1 alone, respectively. Data from two independent experiments showed similar results and were summarized. **C,** IgE-facilitated T cell activation was blocked by pre-incubation of B cells with an anti-CD23 antibody (anti-CD23) but not by an isotype control antibody (IgG_1).

Table E1. Expression of IgD and CD27 on CD23 positive and CD23 negative B cells. The mean percentage of CD23 positive and negative cells was investigated in total B cells as well as among naïve (CD27 negative, IgD positive), post-switch memory (CD27 positive, IgD negative), resting memory (IgD- CD27-) and pre-switch memory (IgD+ CD27+) B cells. Ranges are shown in parentheses.

Table E2. Expression of surface markers on sorted EBV-transformed B cells. B cells sorted according to their CD23 expression (high, intermediate, low) were stained for expression of CD19, HLA class II (HLA-DR) and co-stimulatory molecules CD80 and CD86. MFI: mean fluorescence intensity.

FIG E1: CD23-positive B cells mainly have a naïve IgD positive phenotype. Data from one representative patient are shown. Of the 78.1% CD23-positive B cells, 94.4% were IgD positive, naïve cells. The population of CD23-negative B cells consisted of 21.9% of the cells of which 49.6% were IgD positive, naïve cells and 9.17% were CD27 positive, IgD negative memory B cells.

FIG E2: Changes of CD23 surface density on B cells after addition of IgE. Shown are the percentage changes of CD23 density on B cells in PBMC from allergic (black)

619 and non-allergic (white) subjects cultured after addition of a monoclonal IgE antibody
620 (1 µg/ml IgE) for 6 days as compared to untreated cells.

621

622

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758

TABLE I. Demographic and clinical data of grass pollen allergic patients

			current grass pollen symptoms	other allergen	total IgE	grass pollen	SPT
Patient	Sex	Age	conjunctivitis (c), rhinitis (r), asthma (a), dermatitis (d)	sources	[kU/L]	IgE [kUA/L]	[mm^2]
1	f	28	r	a, h ,t	77	3.3	72.4
2	m	34	c, r	a, f, m, t	94	33.1	14.8
3	m	26	c, r, a	a, f, h, t, w	462	22.5	101.7
4	m	30	c, r	-	33	14.6	20.6
5	f	29	c, r	t, w	395	82.6	17.4
6	m	44	c, r, d	-	82	5.9	61.2
7	m	34	c, r, a, d	a, f, t	680	19.7	34.2
8	m	22	c, r, a, d	a	740	167.0	55.7
9	m	30	c, r, d	a, h, t	10856	1204.0	238.7
10	f	31	c, r, a, d	f, h, t	4967	338.0	169.5
11	f	18	c, r, d	a, h, t, w	1249	92.3	61.9
12	f	32	c, r, a, d	a	695	31.9	20.4
13	f	29	c, r, a, d	a, t	110	19.7	58.4
14	m	41	a, d	a, f, t, w	649	93.8	119.7
15	f	24	r, a, d	a, t, f	346	33.8	159.3
16	m	40	r, d	a, h, t	9113	222.0	78.5
17	f	28	c, r	h, t	277	1.1	12.6
				Median	**462**	**33**	**61**

-: no other allergen source, a: animal dander, f: food, h: house dust mite, m: mould, t: tree pollen, w: weed pollen

TABLE IIA. Percentages of CD23-positive cells among different cell types

	Mean (SD) n=17	Range n=17
T cells	0.02 (0.02)	0-0.07
NK cells	0.57 (0.97)	0-3.35
Monocytes	1.38 (1.89)	0-7.88
Neutrophils	0.00 (0.00)	0.00
Platelets	0.09 (0.13)	0-0.54
Dendritic cells	0.43 (0.97)	0-4
Eosinophils	0.04 (0.07)	0-0.23
Basophils	0.00 (0.00)	0.00
B cells	49.81 (21.47)	3.3-82.33

SD: standard deviation

Table IIB. Numbers of CD23 molecules per cell and concentrations of soluble CD23 in serum

Patient	CD23 molecules per B cell	CD23 molecules per monocyte	soluble CD23 (ng/ml)
1	4869	n.d.	1.2
2	2168	n.d.	0.9
3	3183	n.d.	1.8
4	3088	n.d.	1.6
5	2497	1230	1.0
6	2758	1228	2.3
7	2327	1465	1.1
8	3552	n.d.	1.3
9	6539	n.d.	0.9
10	6719	388	1.1
11	5749	n.d.	1.5
12	2930	1399	1.9
13	3278	826	1.5
14	3989	n.d.	1.0
15	3125	n.d.	1.3
16	6530	n.d.	2.1
17	4150	n.d.	1.3
Mean	3968	1089	1.4

n.d.: not done

FIG 1.

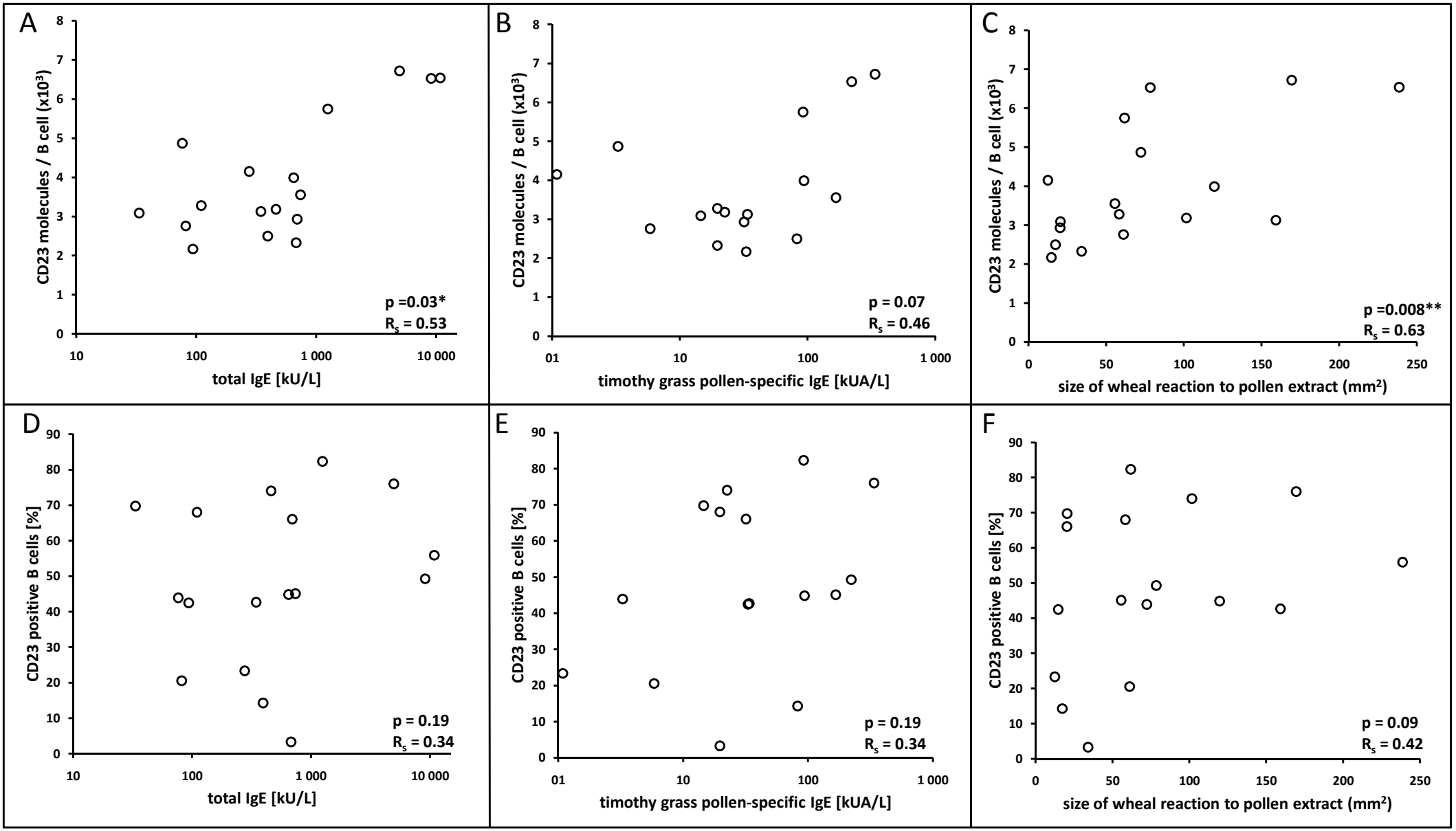
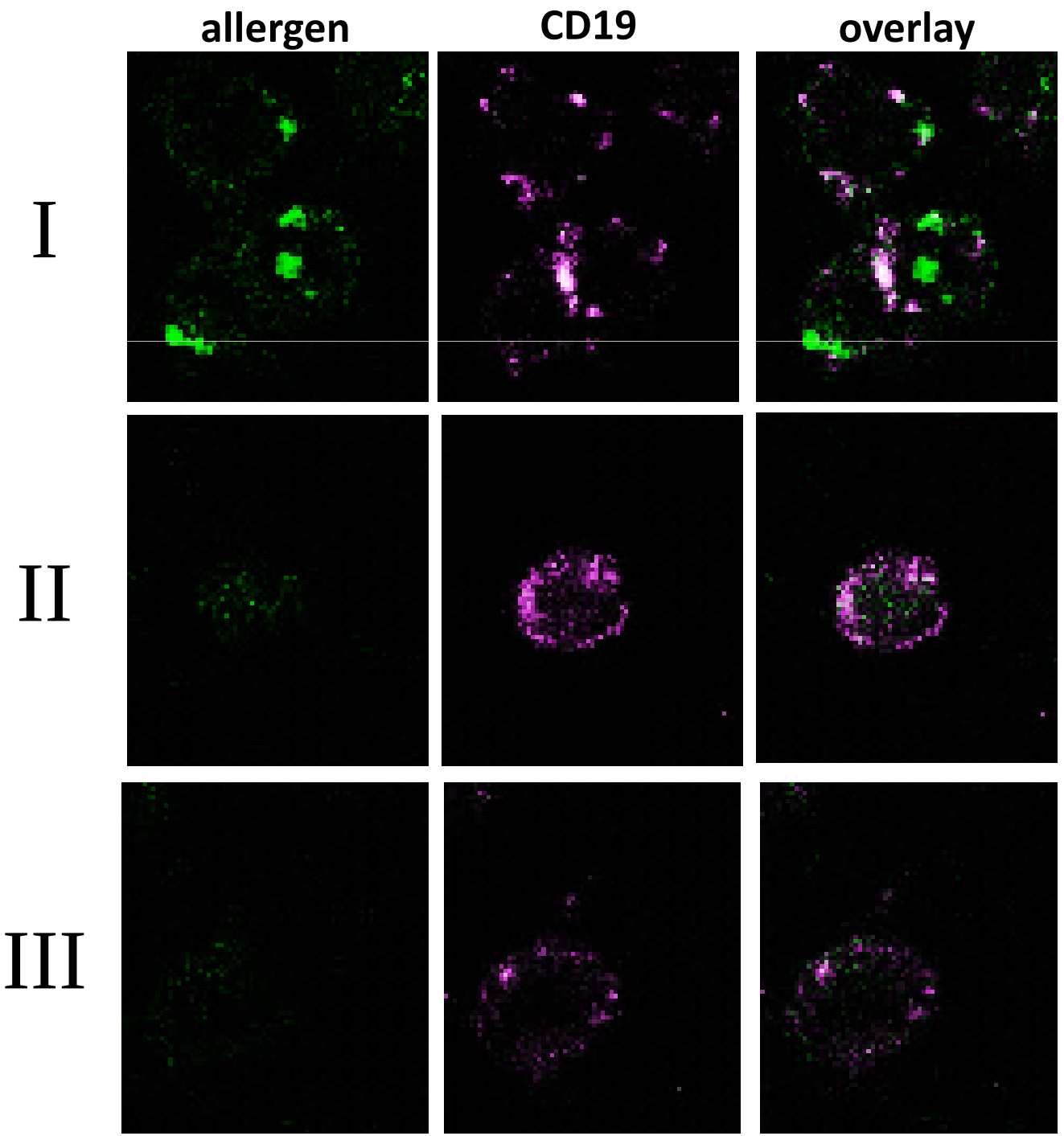
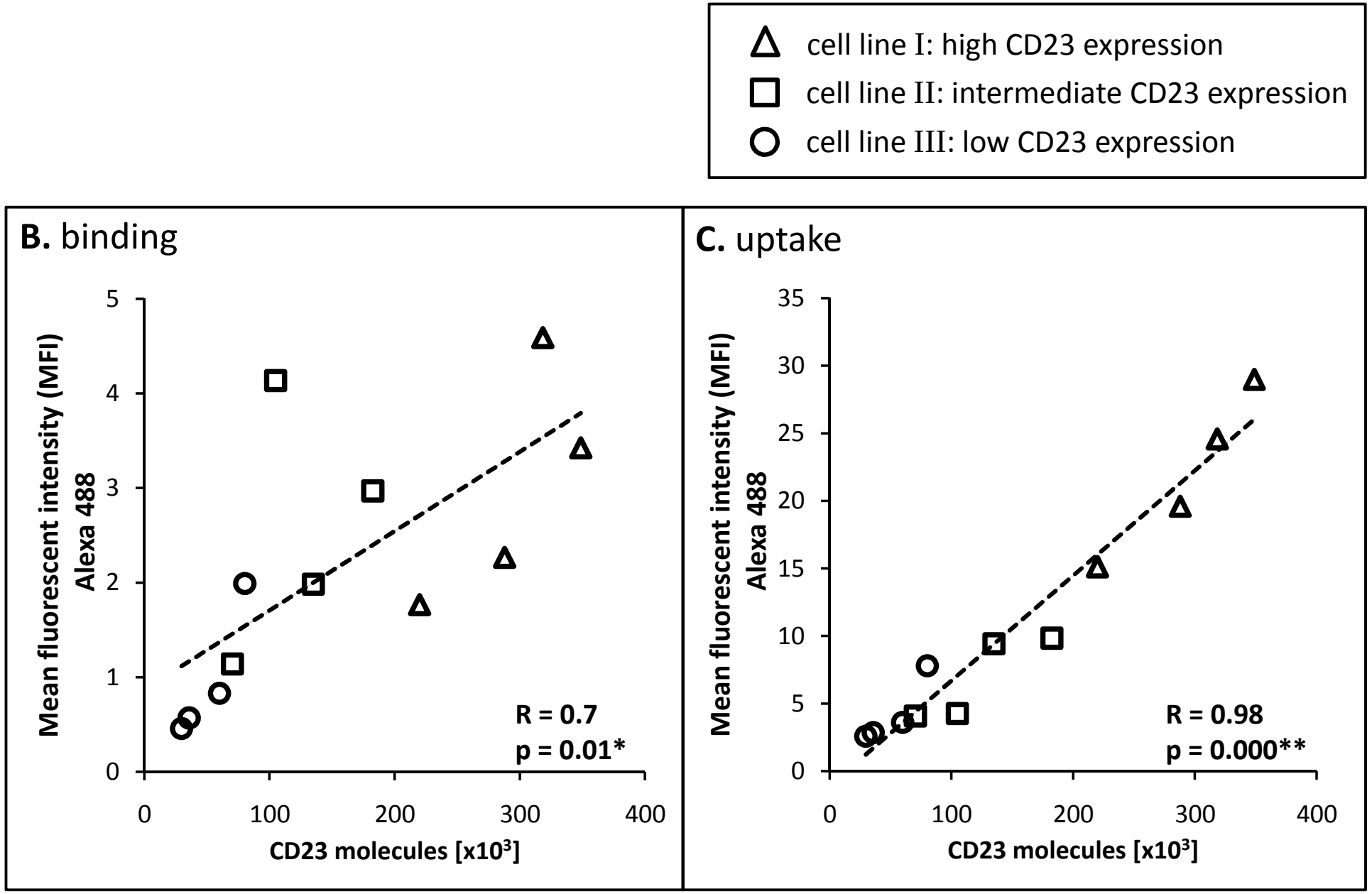

A
CD23 molecules / B cell (x10³)
total IgE [kU/L]
p =0.03*
R_s = 0.53
B
CD23 molecules / B cell (x10³)
timothy grass pollen-specific IgE [kUA/L]
p = 0.07
R_s = 0.46
C
CD23 molecules / B cell (x10³)
size of wheal reaction to pollen extract (mm²)
p =0.008**
R_s = 0.63
D
CD23 positive B cells [%]
total IgE [kU/L]
p = 0.19
R_s = 0.34
E
CD23 positive B cells [%]
timothy grass pollen-specific IgE [kUA/L]
p = 0.19
R_s = 0.34
F
CD23 positive B cells [%]
size of wheal reaction to pollen extract (mm²)
p = 0.09
R_s = 0.42

FIG 2.

A.




cell line I: high CD23 expression
cell line II: intermediate CD23 expression
cell line III: low CD23 expression
B. binding
Mean fluorescent intensity (MFI) Alexa 488
CD23 molecules [x10³]
R = 0.7
p = 0.01*
C. uptake
Mean fluorescent intensity (MFI) Alexa 488
CD23 molecules [x10³]
R = 0.98
p = 0.000**

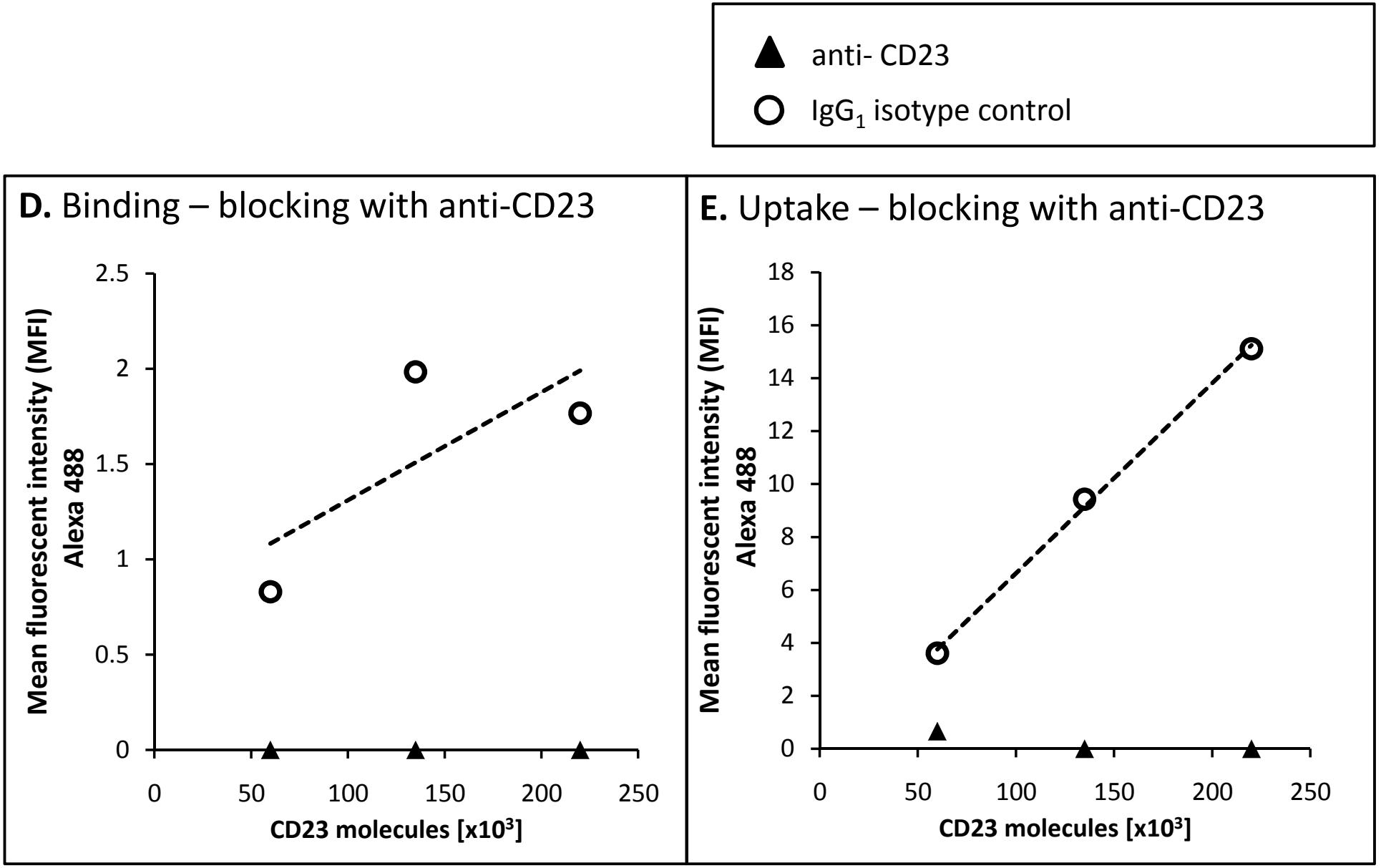
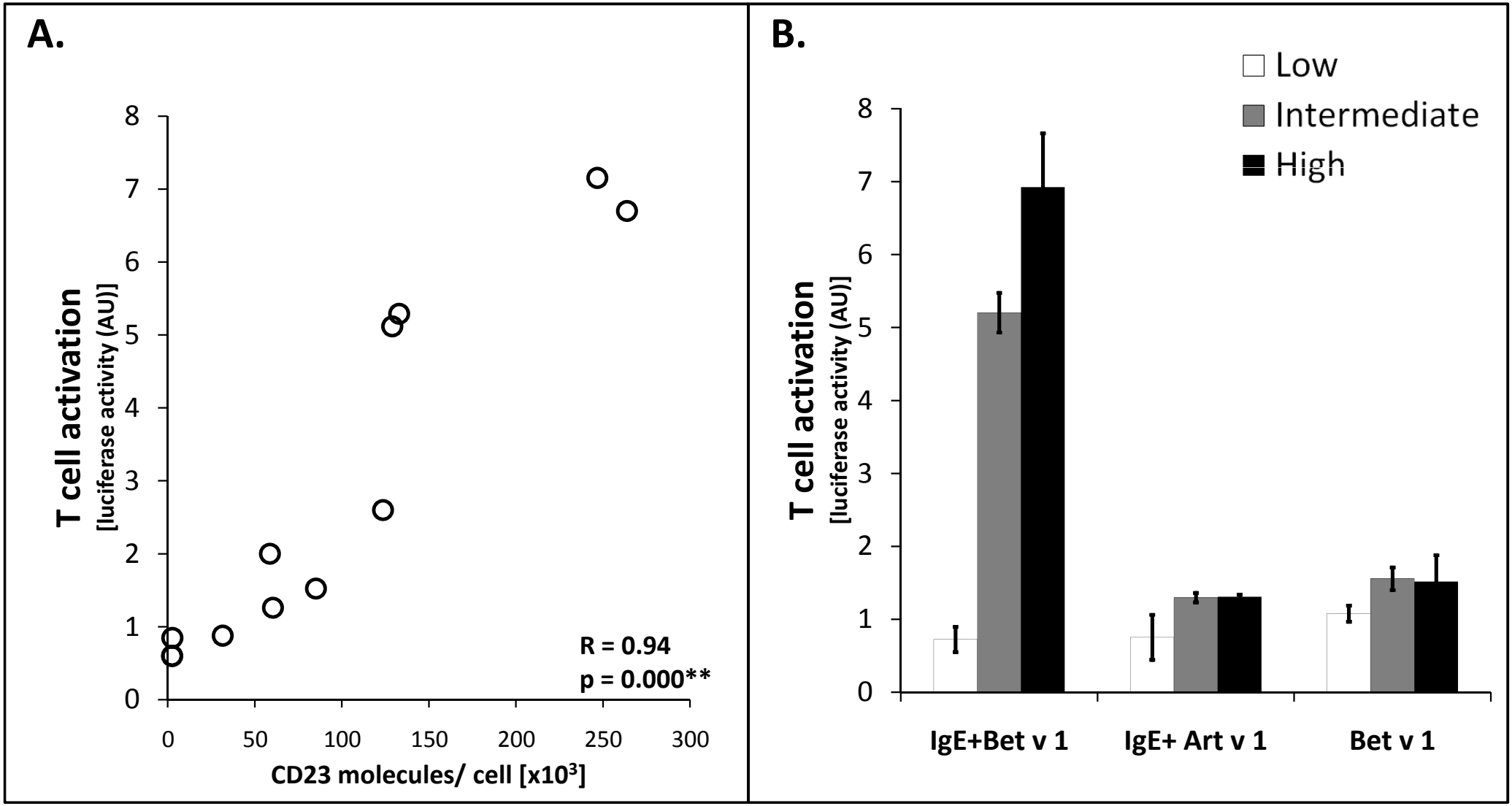

anti- CD23
IgG1 isotype control
D. Binding – blocking with anti-CD23
Mean fluorescent intensity (MFI) Alexa 488
2.5
2
1.5
1
0.5
0
0
50
100
150
200
250
CD23 molecules [x10³]
E. Uptake – blocking with anti-CD23
Mean fluorescent intensity (MFI) Alexa 488
18
16
14
12
10
8
6
4
2
0
0
50
100
150
200
250
CD23 molecules [x10³]

FIG 3.


A.
T cell activation
[luciferase activity (AU)]
0
1
2
3
4
5
6
7
8
0
50
100
150
200
250
300
CD23 molecules/ cell [x10³]
R = 0.94
p = 0.000**
B.
Low
Intermediate
High
T cell activation
[luciferase activity (AU)]
IgE+Bet v 1
IgE+ Art v 1
Bet v 1

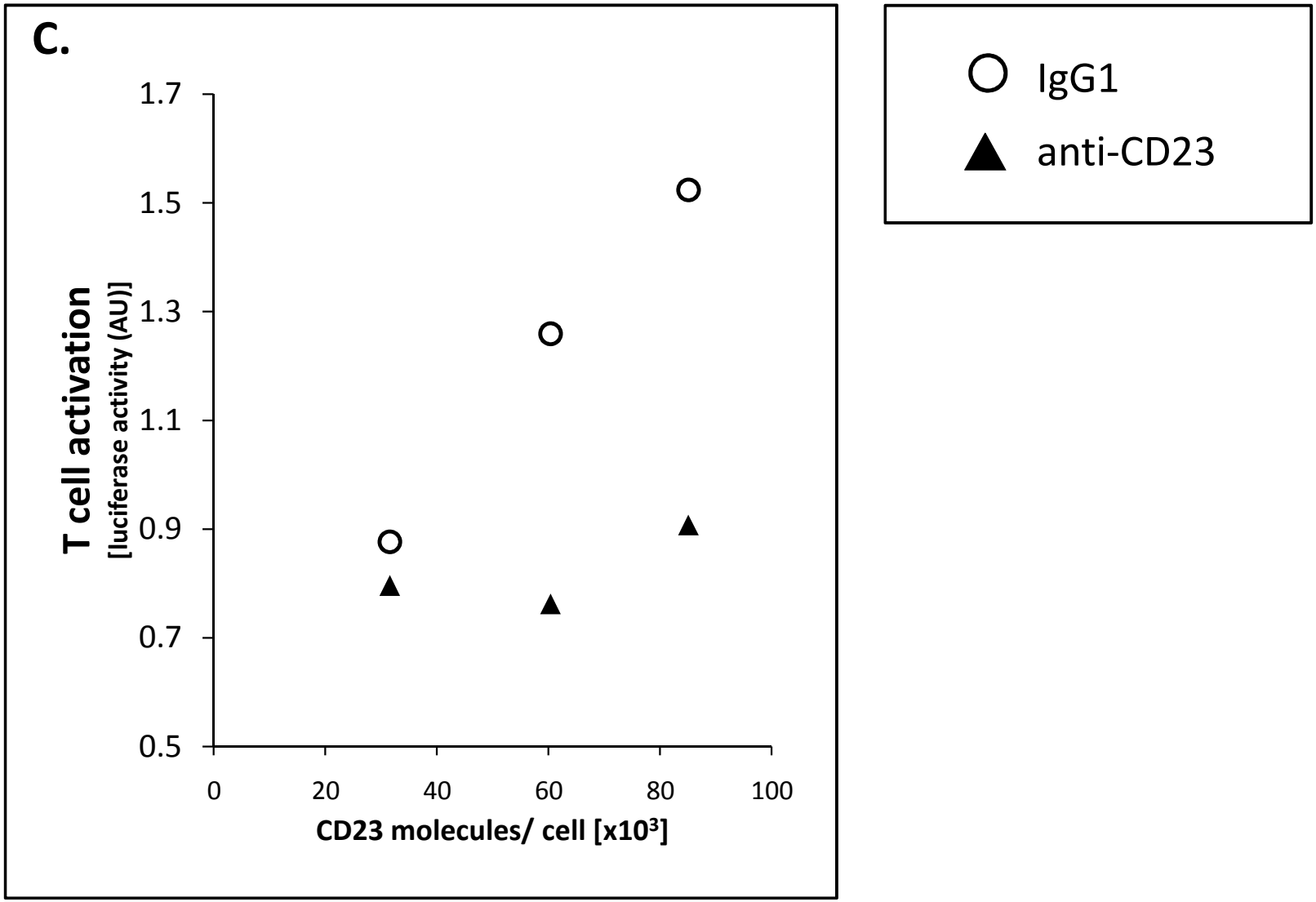

C.
1.7
1.5
1.3
1.1
0.9
0.7
0.5
T cell activation
[luciferase activity (AU)]
0
20
40
60
80
100
CD23 molecules/ cell [x10³]
IgG1
anti-CD23

Table E1. Expression of IgD and CD27 on CD23 positive and CD23 negative B cells

		CD23 positive	CD23 negative
total B cells	CD19+	**65.1** (55.8-77.9)	**34.9** (22.1-44.2)
naive B cells	IgD+ CD27-	**89.2** (81.8-94.5)	**42.9** (31.9-42.9)
post-switch memory B cells	IgD- CD27+	**1.1** (0.25-1.72)	**8.9** (6.9-10.2)
resting memory B cells	IgD- CD27-	**9.2** (4.8-15.9)	**47.1** (41.2-57.3)
pre-switch memory B cells	IgD+ CD27+	**0.5** (0.5-0.6)	**1.1** (0.6-1.5)

Table E2. Expression of surface markers on sorted B cells

	Mean fluorescent intensity (MFI)		
	High	**Intermediate**	**Low**
CD23	325	138	11
CD19	68	48	17
HLA class II	271	271	175
CD80	231	139	34
CD86	793	540	204

FIG E1.

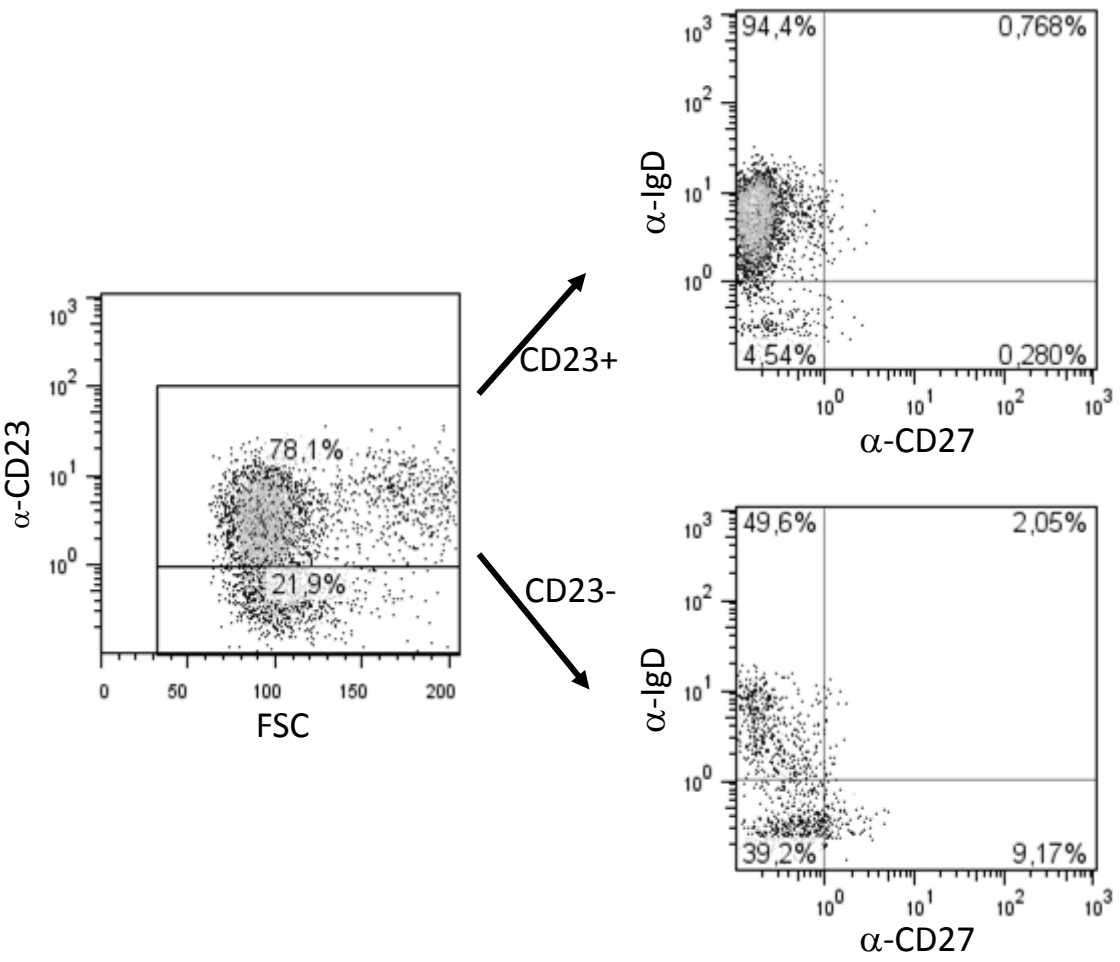
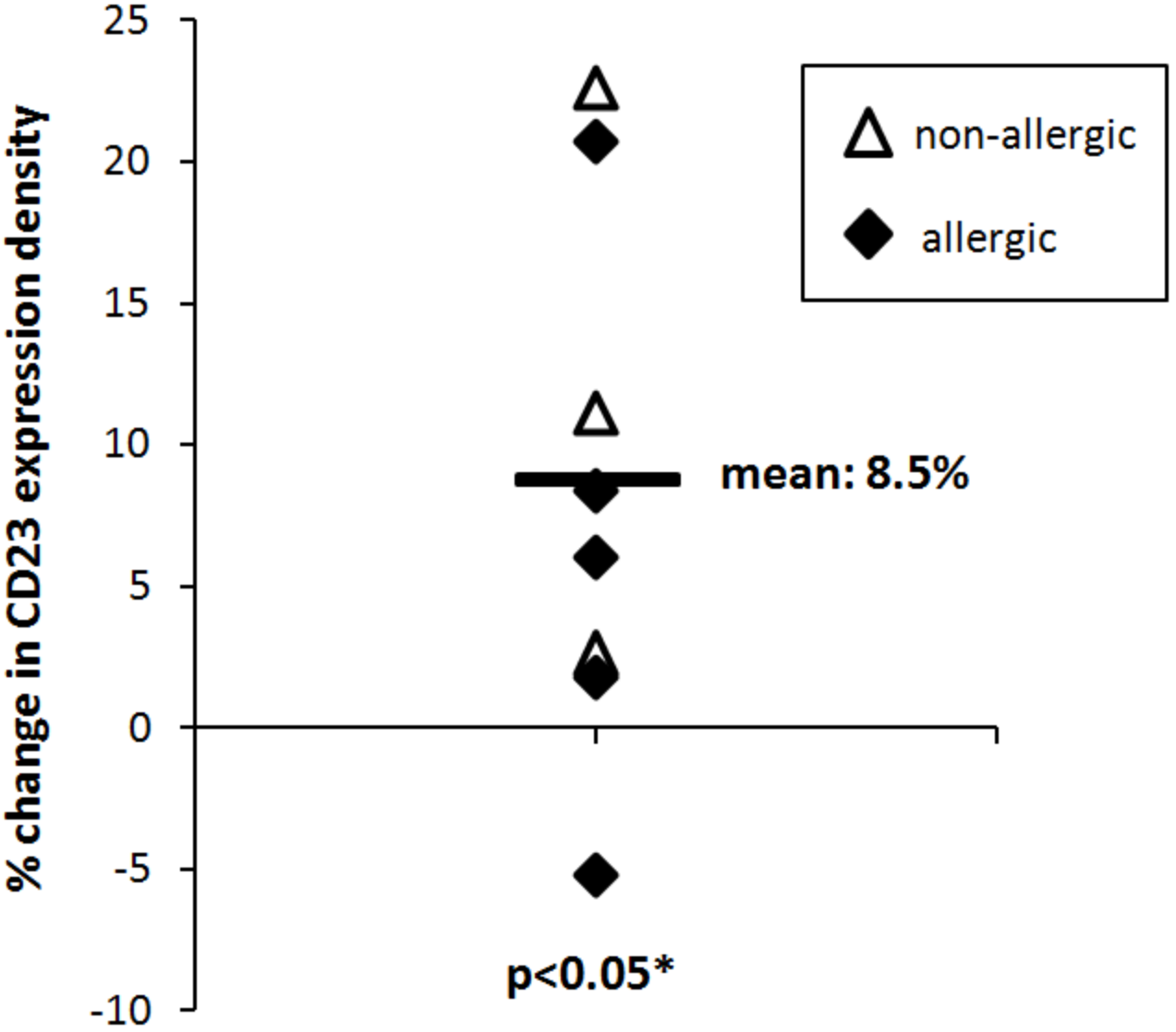

α-CD23
FSC
78,1%
21,9%
CD23+
CD23-
α-IgD
α-CD27
94,4%
0,768%
4,54%
0,280%
49,6%
2,05%
39,2%
9,17%

FIG E2


25
20
15
10
5
0
-5
-10
% change in CD23 expression density
non-allergic
allergic
mean: 8.5%
p<0.05*