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IgG Antibodies in Food Allergy Influence Allergen–Antibody Complex Formation and Binding to B Cells: A Role for Complement Receptors

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Allergen–IgE complexes are more efficiently internalized and presented by B cells than allergens alone. It has been suggested that IgG Abs induced by immunotherapy inhibit these processes. Food-allergic patients have high allergen-specific IgG levels. However, the role of these Abs in complex formation and binding to B cells is unknown. To investigate this, we incubated sera of peanut- or cow's milk-allergic patients with their major allergens to form complexes and added them to EBV-transformed or peripheral blood B cells (PBBCs). Samples of birch pollen-allergic patients were used as control. Complex binding to B cells in presence or absence of blocking Abs to CD23, CD32, complement receptor 1 (CR1, CD35), and/or CR2 (CD21) was determined by flow cytometry. Furthermore, intact and IgG-depleted sera were compared. These experiments showed that allergen–Ab complexes formed in birch pollen, as well as food allergy, contained IgE, IgG1, and IgG4 Abs and bound to B cells. Binding of these complexes to EBV-transformed B cells was completely mediated by CD23, whereas binding to PBBCs was dependent on both CD23 and CR2. This reflected differential receptor expression. Upon IgG depletion, allergen–Ab complexes bound to PBBCs exclusively via CD23. These data indicated that IgG Abs are involved in complex formation. The presence of IgG in allergen–IgE complexes results in binding to B cells via CR2 in addition to CD23. The binding to both CR2 and CD23 may affect Ag processing and presentation, and (may) thereby influence the allergic response. *The Journal of Immunology*, 2013, 191: 000–000.

Depending on the Ag/Ab ratio in the circulation, immune complexes may be formed upon exposure to an Ag. These formed complexes play an important role in Ag presentation. They bind to leukocytes via Fc and/or complement

receptors (CRs), after which they are internalized and processed to facilitate Ag presentation.

Previous research has shown that Ag–Ab complexes formed after vaccination contained complement factors and bound to B cells via CR1 (CD35) and CR2 (CD21). These complexes led to more efficient Ag presentation to T cells than complexes without complement (1, 2). In addition, studies with Ag artificially coupled to complement components indicated that these complexes activated T cells at lower Ag concentrations compared with free Ag (3, 4).

Also in allergy, Ag–Ab complex formation has been observed. These allergen–IgE complexes were able to bind to B cells via the low-affinity IgE receptor CD23 (5–7). As with the vaccination studies, complex formation enhanced the Ag uptake and presentation of allergens (5, 6). Furthermore, studies have shown that this IgE-facilitated Ag presentation (IgE-FAP) induced more Th2 skewing (8, 9). As a model system for IgE-FAP, most studies used EBV-transformed B cells (EBV-B cells), which have a high expression of CD23.

So far, the role of IgE-FAP in allergy has mainly been investigated for inhalation allergens, such as birch pollen, grass pollen, and house dust mite (6, 7, 10, 11). Only one study has investigated IgE-FAP in food allergy (FA), that is, peanut allergy (PA) (12). In line with other studies, this study revealed that, in the presence of specific IgE, peanut-specific T cells are activated at lower allergen concentrations. In PA, not only specific IgE levels, but also specific IgG levels are elevated (13, 14). This was also demonstrated for cow's milk allergy (CMA) (15). However, the role of these specific IgG Abs in allergen–Ab complex formation in allergy is unclear.

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Abbreviations used in this article: BPA, birch pollen allergy; CMA, cow's milk allergy; CR, complement receptor; EBV-B cells, EBV-transformed B cells; FA, food allergy; HA-IgG, heat-aggregated IgG; HC, healthy control subject; IgE-FAP, IgE-facilitated Ag presentation; MFI, median fluorescence intensity; PA, peanut allergy; PBBCs, peripheral blood B cells.

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One study showed that allergen–Ab complexes containing IgE, IgG1, and/or C1q can be formed in the circulation upon a challenge with cow's milk in CMA patients, which suggests that IgG Abs may be involved in complex formation (16). In contrast, other studies have suggested that IgG Abs block complex formation and thereby reduce Ag presentation. In patients treated with allergen immunotherapy, specific IgG levels, especially IgG4, were increased, whereas binding of allergen–IgE complexes to EBV-B cells and subsequent T cell activation were reduced (7, 10, 11, 17, 18). Additional IgG-depletion experiments indicated that IgG Abs were involved in the inhibition of complex binding and Ag presentation (7, 10, 11).

In this study, the formation of allergen–Ab complexes and their binding to B cells in FA as compared with birch pollen allergy (BPA) was investigated. Complexes were stained for the presence of IgE, IgG1, and IgG4 to determine their composition. Binding to EBV-B cells and to B cells within freshly isolated PBMCs incubated with or without blocking Abs for several receptor candidates, that is, CD23, the low-affinity IgG receptor FcγRII (CD32), CR1, and CR2, was examined. In addition, IgG Abs were depleted from the serum to investigate their role in complex formation and binding.

Materials and Methods

Patients

Fifteen CMA patients (age, 26–68 y; median, 39 y), 15 PA patients (age, 20–37 y; median, 23 y), 15 BPA patients (age, 18–60 y; median, 39), and 4 healthy control subjects (HCs; age 25–59 y, median 27) were included in this study. The diagnoses of CMA, PA, and BPA were based on a suggestive history, a positive double-blind, placebo-controlled food challenge, positive IgE specific for cow's milk, peanut, or birch pollen (determined by CAP system FEIA; Thermo Fisher Scientific, Uppsala, Sweden), and/or a positive skin prick test. After informed consent was obtained, venous blood samples were taken from the patients and the control subjects. Plasma/serum was collected and stored at –20°C until further use. This study was approved by the Ethics Committee of the University Medical Center Utrecht.

Allergens

Cow's milk protein and purified αS1-casein (purity >95%) were obtained from Nizo Food Research (Ede, The Netherlands). Crude peanut extract and purified Ara h 2 (purity >95%) were a kind gift from TNO Innovation for Life (Zeist, The Netherlands). Birch pollen extract was obtained from ALK Abellø (Hørsholm, Denmark), whereas recombinant Bet v 1 (expressed in *Escherichia coli*, purity >98%) was purchased at Biomay (Vienna, Austria).

Antibodies

HRP-conjugated goat anti-human IgE (1:10,000) was purchased from KPL (Gaithersburg, MD). HRP-conjugated mouse anti-human IgG1 (1:20,000) and -IgG4 (1:30,000) were acquired from Sanquin (Amsterdam, The Netherlands). Unlabeled mouse IgG isotype control (DAK-GO1, 1:2), and PE-labeled and unlabeled mouse-anti-human CD23 (clone MHM6, 1:10) were obtained from Dako Denmark A/S (Glostrup, Denmark). PE-labeled and unlabeled mouse anti-human CD32 (clone AT10, 1:10) were purchased from AbD Serotec (Martinsried, Germany). PE-labeled mouse IgG1 isotype control (clone MOPC-31C, 1:5), mouse anti-human CR1 (clone E11, 1:10) and CR2 (clone B-ly4, 1:10), streptavidin (1:450), unlabeled mouse anti-human CR2 (clone 1048, 1:5), biotinylated mouse anti-human IgE, IgG1, IgG4 (all 1:250), and FITC-labeled mouse anti-human CD20 (clone 2H7, 1:10) were all acquired from BD Biosciences (San Diego, CA). Unlabeled mouse anti-human CR1 (clone J3D3, 1:50) was bought at Beckman Coulter (Brea, CA).

Allergen-specific IgE, IgG1, and IgG4 ELISA

ELISA to determine allergen-specific IgE, IgG1, and IgG4 levels to cow's milk protein, αS1-casein, crude peanut extract, Ara h 2, birch pollen extract, or Bet v 1 was performed as described previously (19). Results are expressed as arbitrary units per milliliter.

Cells

EBV-B and IIA1.6 cells (murine cell line expressing human CD32) were cultured in RPMI 1640 + GlutaMAX-I supplemented with 10% v/v heat-

inactivated FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA). PBMCs were isolated from heparinized venous blood samples from healthy donors by density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare Life Sciences, Little Chalfont, U.K.).

Allergen–Ab complex binding to B cells

Complex binding to B cells was investigated as described previously with some adjustments (20). In short, plasma (for 21 subjects) or serum (for 4 subjects) of 25 subjects ($n = 7$ for each allergy group and $n = 4$ for the healthy subjects; see Supplemental Table I) was incubated for 1 h at 37°C with several concentrations αS1-casein, Ara h 2, or Bet v 1 (range from 0.001 to 100 µg/ml) in PBS-2% w/v human serum albumin (Sanquin) to form allergen–Ab complexes. Subsequently, 4.5×10^5 EBV-B cells or freshly isolated PBMCs were added to the samples and incubated for 1 h at 37°C to allow complexes to bind to the cells. After 1 h, samples were washed, divided over three tubes, and stained for binding of allergen–Ab complexes with biotinylated mouse anti-human IgE, IgG1, or IgG4 Abs for 30 min at 4°C, followed by a PE-labeled streptavidin staining. Simultaneously, peripheral blood B cells (PBBCs) within the PBMCs were stained with FITC-labeled mouse anti-human CD20. Fluorescence was measured with flow cytometry (FACSCanto II; BD Biosciences, Franklin Lakes, NJ).

To evaluate receptors involved in binding of allergen complexes, we preincubated the cells with unlabeled blocking Abs against CD23, CD32, CR1, CR2, or a mouse IgG isotype control for 30 min at 4°C before they were incubated with the plasma/serum mixtures. Optimal Ab dilutions for receptor blocking were determined by titration. The role of IgG Abs in the formation and subsequent binding of allergen complexes to the cells was assessed by depleting the Abs from plasma/serum using ProteoPrep Immunoaffinity Albumin & IgG Depletion kit (Sigma-Aldrich, Saint Louis, MO) according to the manufacturer's instructions with a small adjustment. Undiluted serum/plasma samples were used instead of prediluted samples. Because samples were diluted twice during IgG depletion, control samples were diluted to the same extent in these experiments.

CD23, CD32, CR1, and CR2 expression on B cells

To determine receptor expression on EBV-B cells and PBMCs, we incubated 1×10^5 cells with PE-labeled mouse anti-human CD23, CD32, CR1, CR2, or mouse IgG1 isotype control for 30 min at 4°C. To distinguish PBBCs within the PBMCs, we costained PBBCs with FITC-labeled mouse anti-human CD20. The fluorescence was measured by flow cytometry.

Binding of heat-aggregated IgG to B cells

To investigate whether IgG complexes are capable of binding to CD32 and to determine the effect of complement activation on this binding, 500 µg/ml heat-aggregated IgG (HA-IgG) in PBS-2% w/v human serum albumin was incubated with or without serum from an HC. Subsequently, 4.5×10^5 EBV-B cells, IIA1.6 cells, or freshly isolated PBMCs were added to the samples and incubated for 1 h at 37°C to allow aggregates to bind to the cells. Bound IgG Abs were stained as described earlier. To determine which receptors were involved in the binding, we preincubated cells with blocking Abs to CD23, CD32, CR1, and CR2 as described earlier.

Statistical analyses

All data were analyzed with GraphPad Prism version 5.0d for Macintosh (GraphPad Software, San Diego, CA). The median fluorescence intensities (MFIs) of the receptors and of the IgE/IgG/IgG4 binding were corrected for the background staining of the isotype. Negative values were fixed to 1. The MFI of the receptors and the ELISA data were analyzed using the Kruskal–Wallis method followed by a Dunn's post hoc test for selected groups. For blocking and IgG-depletion experiments, the corrected MFIs were log-transformed and analyzed using repeated-measures ANOVA with a Bonferroni's multiple-comparison posttest for selected groups. The p values <0.05 were considered significant.

Results

High IgG levels in FA

Allergen-specific IgE, IgG1, and IgG4 levels in BPA, PA, and CMA patients were measured by ELISA (Fig. 1). IgE levels were comparable among the different groups. In contrast, in PA and

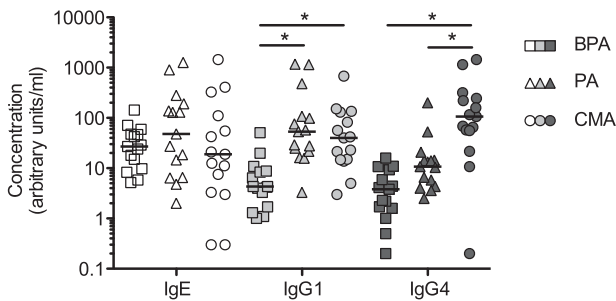


FIGURE 1. Allergen-specific IgE, IgG1, and IgG4 levels in BPA, PA, and CMA. Birch pollen, peanut or cow's milk-specific IgE, IgG1, and IgG4 levels were determined in BPA, PA, or CMA patients, respectively. Each patient is represented by a square, triangle, or circle in the scatterplot. Lines indicate median values. IgE, IgG1, and IgG4 levels between allergies were compared ($n = 15$). * $p < 0.05$.

CMA patients, allergen-specific IgG1 levels (median, 53.1 [range, 3.3–1170] and 40.1 [range, 3–678.5] arbitrary units/ml, respectively) were significantly higher than those in BPA patients (median, 4.3; range, 1–50.8). Furthermore, CMA patients had significantly higher levels of allergen-specific IgG4 (median, 106.4; range, 0.2–1440) than PA and BPA patients (median, 10.7 [range, 2.5–200.6] and 3.8 [range, 0.2–16], respectively). Seven patient samples from each group were used for in vitro complex formation experiments. In addition, samples of four HCs, which were selected based on high IgG1/IgG4 levels specific for α S1-casein in the absence of IgE, were used for these experiments. The specific Ab levels to the major allergens in the selected patients and in the HCs are shown in Supplemental Table I.

IgG Abs present in allergen–Ab complexes in BPA and FA

The composition of allergen–Ab complexes binding to EBV-B cells or PBBCs in HCs (Supplemental Fig. 1) and BPA, PA, and CMA patients (Fig. 2, Supplemental Fig. 2) was investigated using Abs for IgE, IgG1, and IgG4.

Immune complexes containing all three isotypes were found to bind to EBV-B cells and PBBCs for BPA patients. Also for PA patients, binding of IgE, IgG1, and IgG4 to both cell types was observed. For CMA patients, complex binding to EBV-B cells was observed in six of the seven patients (CMA 1, 3–7), although complex binding was somewhat lower for two patients (CMA 1, 3). In one patient (CMA 2), hardly any complex binding was observed. Binding of complexes to PBBCs as compared with EBV-B cells was more pronounced. For six CMA patients, complexes binding to PBBCs contained IgE, IgG1, and IgG4, whereas in one patient (CMA 2), complexes contained mainly IgG4 and hardly any IgE or IgG1. The optimal allergen concentration for complex binding was $10\times$ higher for CMA (0.01–1 μ g/ml) than for PA (0.01–0.1 μ g/ml) and BPA patients (0.01–0.1 μ g/ml).

For the HCs, minimal IgG1 and IgG4 binding to PBBCs was seen for three subjects (HC 2–4). In one of the three subjects (HC 2), also minimal IgE binding was found. Hardly any binding to EBV-B cells was observed.

CR2 involved in complex binding to PBBCs

To determine which receptors were involved in the binding of allergen–Ab complexes, EBV-B cells and PBBCs were preincubated with blocking Abs before incubation with the complexes. For each patient, the optimal allergen concentration for the formation of allergen–Ab complexes was used. For BPA, PA, as well as CMA, binding of complexes to EBV-B cells was mainly mediated by CD23 (Fig. 3). In contrast, complex binding to PBBCs was only partially reduced upon blocking this receptor (Fig. 4).

Blocking CD32 had a minor effect on the complex binding to PBBCs for CMA patients and no effect for PA and BPA patients. However, preincubating PBBCs with blocking CR2 Abs did inhibit the binding of complexes to these cells. Moreover, blocking both CD23 and CR2 virtually abrogated the complex binding for BPA and PA patients, and reduced the binding by almost 90% for CMA patients (significant using a t test). Blocking CR1 had only a minor effect on the complex binding to PBBCs. Also, in healthy subjects, the complex binding to PBBCs was mediated by CR2 (data not shown).

Different receptor expression pattern on EBV-B cells compared with PBBCs

To evaluate whether the difference in receptor involvement for EBV-B cells and PBBCs was due to receptor expression, the expression of CD23, CD32, CR1, and CR2 on B cells was determined. Expression of CD23 was much higher on EBV-B cells than PBBCs (Fig. 5). In contrast, CR1 and CR2 expression was significantly lower on EBV-B cells compared with PBBCs, whereas CD32 expression was comparable.

Complement deposition on IgG inhibits binding to CD32 and increases binding to CR1/CR2

The effect of complement deposition on the binding of IgG present in Ag–Ab complexes was investigated by comparing the binding of HA-IgG with or without preincubation with complement-sufficient serum to EBV-B cells, PBBCs, and IIA1.6 cells, of which the last expresses only CD32. Without incubation in serum, binding of HA-IgG to all three cell types was observed and mediated by CD32 (Fig. 6). Upon serum incubation, HA-IgG binding to IIA1.6 cells was strongly reduced, whereas binding to PBBCs was slightly increased. Binding to PBBCs and EBV-B cells was now mediated by CR1 and CR2. In both conditions, HA-IgG binding to EBV-B cells was low compared with PBBCs.

IgG Abs involved in complement activation

To investigate whether IgG Abs were indeed involved in complement activation, IgG Abs were depleted from the plasma samples of PA patients before allergen–Ab complex formation. After IgG depletion, the complexes contained primarily IgE (Fig. 7). The binding of these complexes to PBBCs was completely mediated by CD23, whereas binding to CR2 was negligible.

Discussion

The objective of this study was to investigate allergen–Ab complex formation and binding to B cells in FA in comparison with BPA. Furthermore, the role of specific IgG Abs in these complexes was examined by determining the Ab composition of the complexes and the receptors involved in complex binding.

Confirming previous studies, IgE binding to EBV-B cells was observed for BPA patients (10, 21). Also for PA patients, binding of complexes to these cells was observed, whereas for CMA patients, binding was less pronounced. Interestingly, not only IgE, but also IgG1 and IgG4, were detected in complexes binding to EBV-B cells.

Although EBV-B cells are commonly used in IgE-FAP studies, complex binding to PBBCs presumably represents the in vivo situation better and was therefore also determined. In general, for PA and BPA patients, the binding to PBBCs was comparable with EBV-B cells, whereas for CMA patients, complex binding to PBBCs was more pronounced. The optimal allergen concentration to form complexes was different between the allergies. For CMA, this concentration was $10\times$ higher than for PA and BPA. Because it is known that Ab/allergen ratios influence the complex forma-

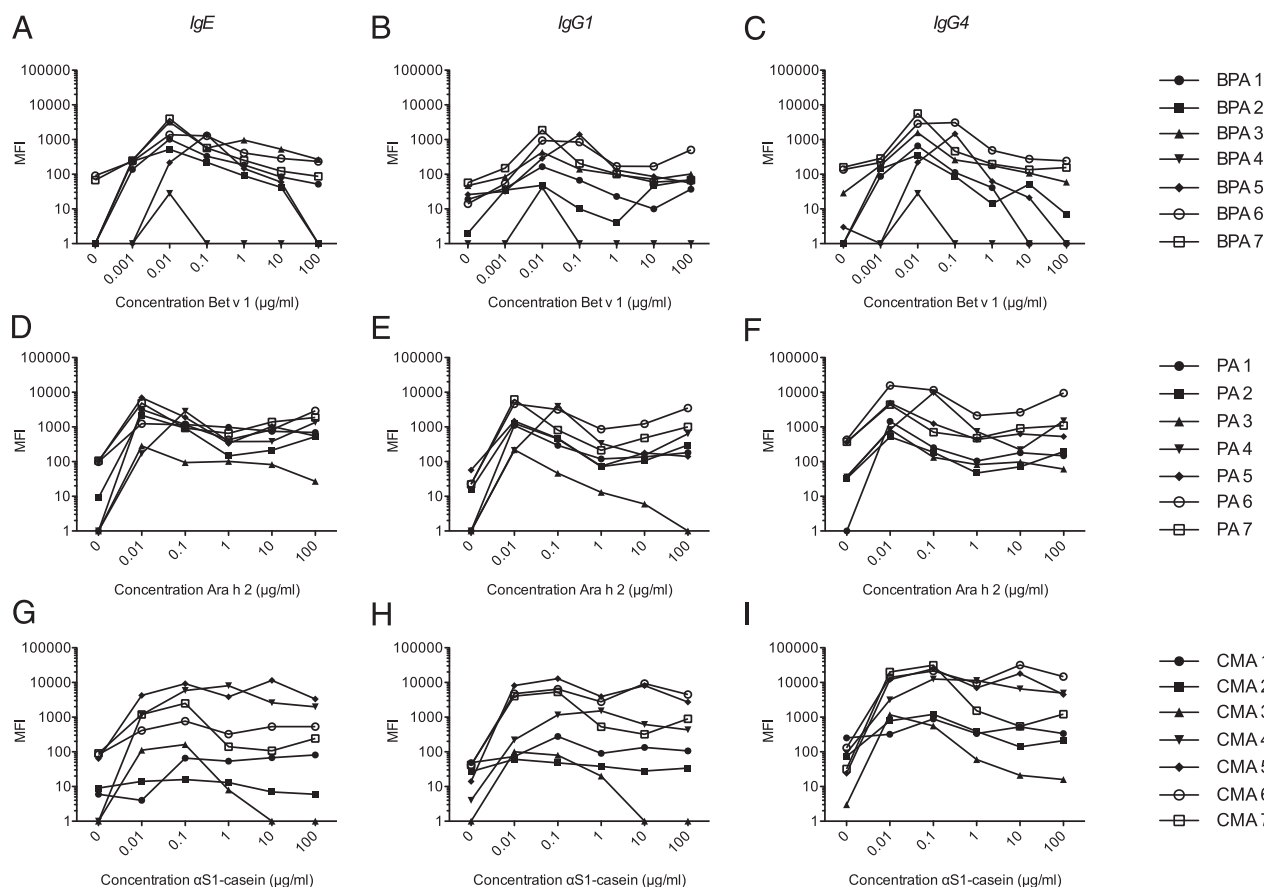


FIGURE 2. Allergen–Ab complex binding to PBBCs. MFI of IgE (**A, D, G**), IgG1 (**B, E, H**), and IgG4 (**C, F, I**) present in complexes binding to PBBCs, after incubation of plasma/serum samples of seven BPA (**A–C**), PA (**D–F**), and CMA (**G–I**) patients and different concentrations of the major allergens Bet v 1, Ara h 2, and α S1-casein, respectively.

tion, the increase in optimal concentration and the less pronounced complex binding for CMA patients compared with BPA and PA patients may reflect the higher absolute Ab levels, mainly because of significantly higher IgG1 and/or IgG4 levels, against the major allergen α S1-casein in the CMA patients.

Previous studies indicated that HCs also have detectable food-specific IgG1 and IgG4 levels (19). To determine whether complex formation and binding also occur in these subjects, we investigated IgE, IgG1, and IgG4 binding to EBV B cells and PBBCs for four HCs. As expected, complex binding was less pronounced for the HCs as for the FA patients. Hardly any Ab binding to the EBV B cells was found, whereas some IgG1 and IgG4 binding to PBBCs was observed for three HCs. Because the allergen-specific IgG1 and IgG4 levels in these subjects are comparable with the levels found in the CMA patients (Supplemental Table I), the less pronounced complex binding probably reflects the lower absolute Ab levels in the HCs because of absence of food-specific IgE levels. Complexes in patients were shown to bind via CD23 and CR2. The data in HCs suggest that absence of IgE in the complexes makes complex binding less efficient. Because of this low complex binding, the functional role of the complexes in HCs may be limited.

Although complex binding to EBV-B cells and PBBCs was comparable, the receptors involved in the binding were different. In accordance with previous studies, blocking CD23 on EBV-B cells inhibited the complex binding for BPA patients completely (6, 7, 22). Also in FA, the binding to EBV-B cells was completely reduced upon blocking CD23. Not only was IgE binding to EBV-B cells inhibited by blocking CD23, but also IgG1 and IgG4

binding, which suggests that the formed complexes contained mixed IgE, IgG1, and IgG4 Abs, and that their binding to EBV-B cells was mediated via IgE. Blocking CD23 on PBBCs reduced complex binding only partially. Surprisingly, the remaining binding was not mediated by CD32, but by CR2.

The differences in the receptors involved in complex binding to EBV-B cells and PBBCs were explained by the expression of these receptors on the cells. As mentioned earlier, EBV-B cells are often used in IgE-FAP as a model system because of their high CD23 expression. However, compared with PBBCs, the expression of CD23 was significantly higher on EBV-B cells, whereas the expression of CR1 and CR2 was lower. The high expression of CD23 probably leads to preferential binding of allergen–Ab complexes via IgE to this receptor. Apparently, the lower expression of CR2 on EBV-B cells minimized the contribution of this receptor to the complex binding, because blocking CD23 completely abrogated the binding to these cells. This is in line with a previous study in which binding of immune complexes to EBV-B cells was observed only to cells with high CR2 expression (23). The authors suggested that complexes bind to EBV-B cells via CR2 when multivalent attachment of the complexes occurs. This hypothesis may also explain why HA-IgG, which is more potent in binding complement components than complexes containing IgE, IgG1, and IgG4, was able to bind to CR2 on EBV-B cells. However, because of the lower CR2 expression, HA-IgG binding to EBV-B cells was low compared with PBBCs. Together, these data show that EBV-B cells may not be a representative model for allergen–Ab complex binding and IgE-FAP *in vivo*.

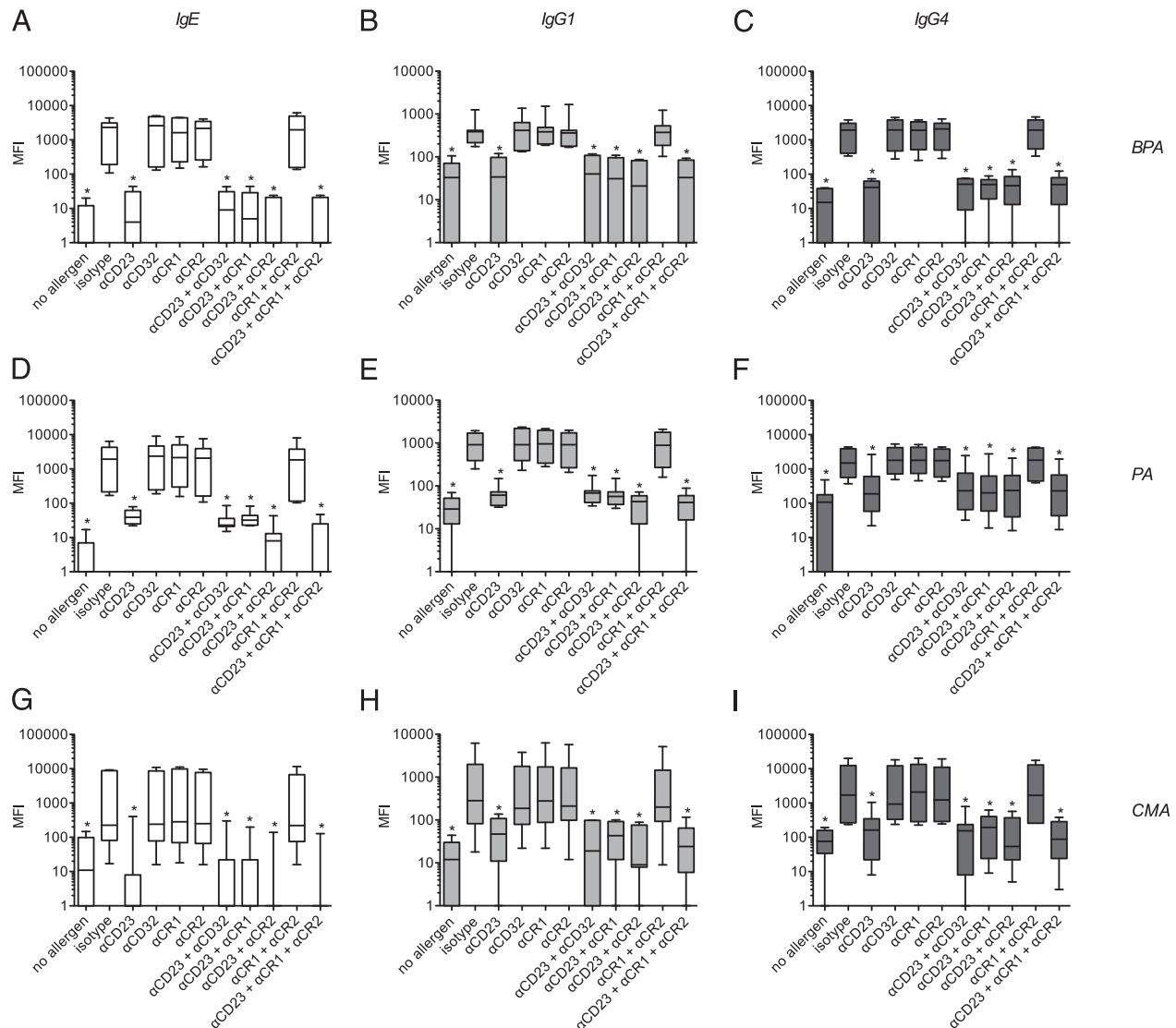


FIGURE 3. Allergen–Ab complex binding after blocking several receptor candidates on EBV-B cells. MFI of IgE (**A**, **D**, **G**), IgG1 (**B**, **E**, **H**), and IgG4 (**C**, **F**, **I**) present in complexes binding to EBV-B cells, with and without CD23, CD32, CR1, and/or CR2 blocking in seven BPA (**A–C**), PA (**D–F**), and CMA (**G–I**) patients. All conditions were compared with isotype control with allergen. * $p < 0.05$.

Immune complexes that contain IgG1 can activate the classical pathway of the complement system. Activation of this system leads to the fixation of complement component C3b to the complexes. This fragment is subsequently degraded to iC3b and C3dg, which both bind to CR2. In contrast with IgG1, IgG4 may inhibit the activation of the classical pathway, or instead may activate the complement system via the alternative pathway (24, 25). However, this activation requires relatively high Ab concentrations and is weaker than that of the classical pathway by IgG1 (25). Thus, IgG, especially IgG1, present in the mixed allergen–Ab complexes may activate the complement system and results in subsequent binding of the complexes to CR2.

IgG was depleted from serum before complex formation to investigate this. Interestingly, binding of complexes formed in serum depleted for IgG was no longer inhibited by blocking CR2, but was completely dependent on CD23. This indicates that complex binding to CR2 on PBBCs was dependent on the presence of IgG Abs in the complexes. Furthermore, in BPA patients with low levels of IgG1, complex binding to PBBCs was mediated by CD23 as described previously, whereas in patients with higher

IgG1 levels, binding occurred via CR2 (data not shown) (11). Also, in PA patients, who all have high IgG1 levels, and in HCs, immune complexes bound to CR2. However, in CMA patients, who have high levels of both IgG1 and IgG4, these effects were less clear. This suggests that, in particular, IgG1 in the complexes was needed for binding to CR2. It would be interesting to deplete IgG1 and IgG4 Abs separately to further investigate the effects of these Abs. In addition, the role of IgG2 and IgG3, which both activate the complement system, needs to be investigated.

Interestingly, previous studies have indicated a direct interaction between CD23 and CR2 (26–28). In addition, they showed that the IgE receptor can bind simultaneously to the CR and IgE. Both membrane and soluble CD23 may be involved in the interaction with CR2. Whereas soluble CD23 is not involved in the complex binding observed in this study, the role of the interaction between membrane CD23 and CR2 is unclear. Because blocking of both CD23 and CR2 has a much stronger blocking effect as compared with blocking CD23 or CR2 alone, our data suggest that the receptors act in a cooperative manner to bind the complexes.

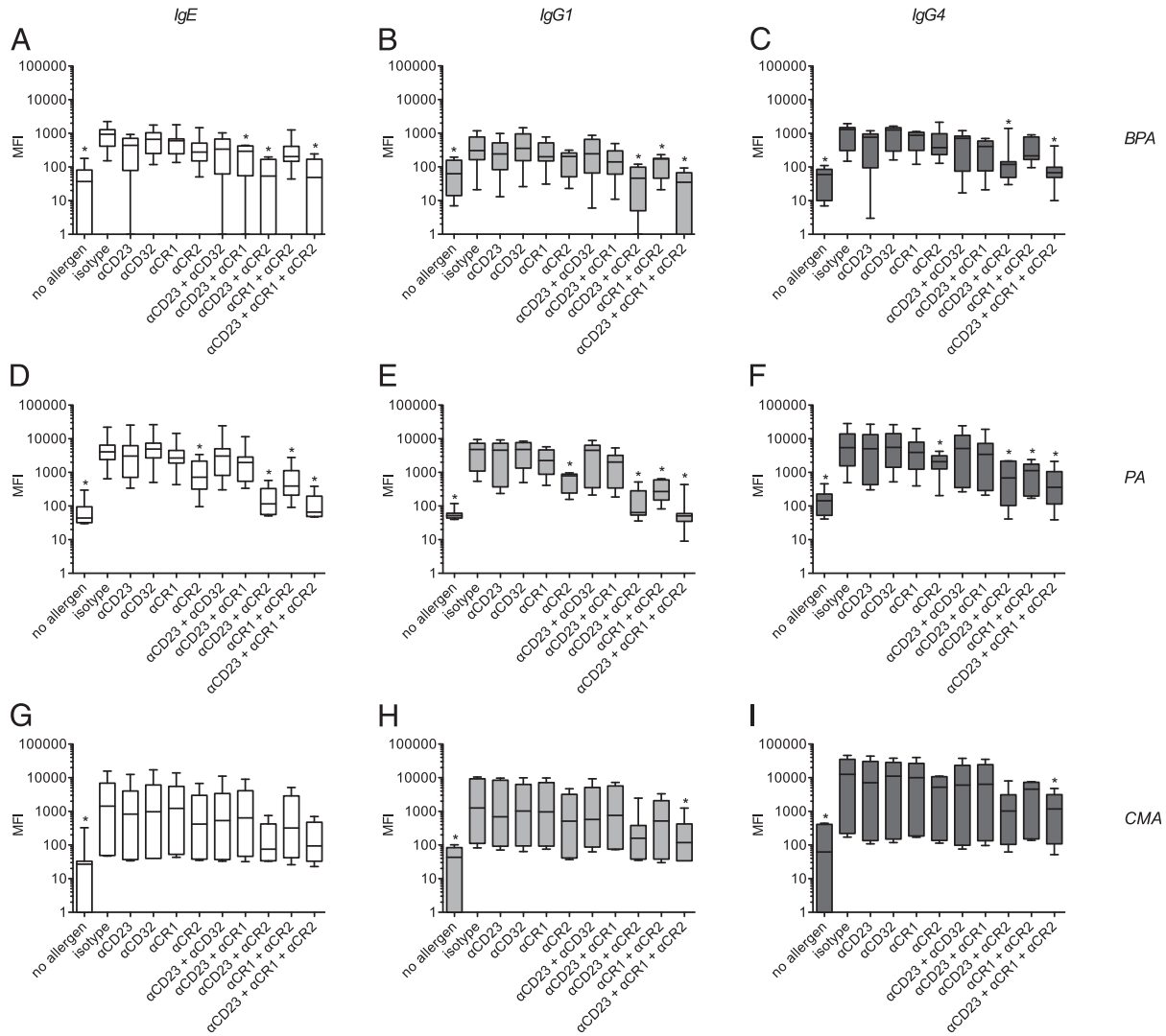


FIGURE 4. Allergen-Ab complex binding after blocking several receptor candidates on PBBCs. MFI of IgE (**A, D, G**), IgG1 (**B, E, H**), and IgG4 (**C, F, I**) present in complexes binding to PBBCs, with and without CD23, CD32, CR1, and/or CR2 blocking in seven BPA (**A–C**), PA (**D–F**), and CMA (**G–I**) patients. All conditions were compared with isotype control with allergen. * $p < 0.05$.

Unexpectedly, CD32 was not involved in the binding of immune complexes to B cells. An obvious explanation for this observation is that because of the presence of IgE and IgG4, the allergen-Ab complexes contained too little IgG1 for binding to CD32. Also, other studies have shown that immune complexes containing other Abs in addition to IgG may not bind to CD32 on PBBCs (1, 2, 22).

However, the experiments with HA-IgG showed that complexes without serum incubation can bind to B cells via CD32, whereas the same complexes incubated with serum bind only via CRs. This points toward another mechanism, that is, that complement fixation to the complexes apparently abrogated binding via CD32. A similar phenomenon was previously described for the binding of IgG1 to another low-affinity IgG receptor, namely, CD16 (29). Moreover, studies investigating the binding sites for FcRs and C3 components on IgG have shown that they bind to the same region (30, 31).

The effect of complement activation on the binding and processing of complexes by B cells has already been investigated in several vaccination studies. In vitro studies showed that complement deposition on complexes formed using sera after vaccination resulted in more efficient uptake and Ag presentation by Ag-specific and nonspecific B cells (1, 2). Moreover, the complement-containing complexes induced an Ab response in Ag-specific B cells, whereas complexes without complement factors did not (32). This is in agreement with in vivo mouse studies, which showed reduced Ab responses in CR1/CR2-deficient mice or in mice treated with CR1/CR2 blocking Abs (33–36). Interestingly, previous research in C3- and CR2-deficient patients has shown that, in particular, IgG4 levels were depressed in these

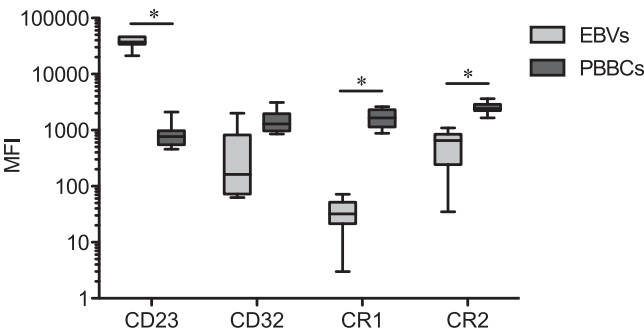


FIGURE 5. Expression of CD23, CD32, CR1, and CR2 on EBV-B cells and PBBCs. The MFI of the different receptors on EBV-B cells and PBBCs is depicted. Expression levels between EBV-B cells and PBBCs were compared ($n = 9$). * $p < 0.05$.

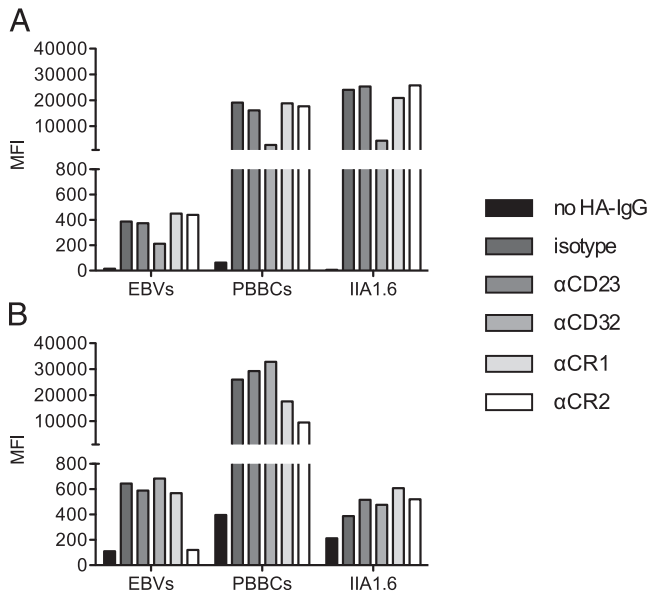


FIGURE 6. Binding of HA-IgG with and without serum incubation to several B cells. Binding of HA-IgG without (**A**) and with (**B**) serum incubation to EBV-B cells, PBBCs, and CD32-expressing IIA1.6 cells preincubated with blocking Abs for CD23, CD32, CR1, or CR2. These figures are representative examples of two experiments.

patients, which may indicate that CR2 activation is important for IgG4 production (37, 38).

The effects of allergen–Ab complexes formed in FA patients on Ag presentation by B cells are still unclear. Although previous IgE–FAP studies have shown that IgG Abs formed after immunotherapy inhibit Ag presentation, these studies were done with EBV-B cells instead of PBBCs (7, 10, 11). Because of differences in receptor expression, complement deposition on complexes has different effects on the binding to receptors on EBV-B cells than on PBBCs. Therefore, we investigated the effect of complex formation in FA patients on Ag presentation by both EBV-B cells and

PBBCs in a preliminary study. As in previous IgE–FAP studies (7, 10, 11), these preliminary experiments showed that T cell proliferation occurred at a lower concentration when incubating EBV cells with complexes from serum/plasma of BPA and CMA patients, incubated with increasing concentrations of Bet v 1 and α S1 casein, respectively (Supplemental Fig. 3). In addition, depleting IgG Abs from the serum/plasma of CMA patients increased the Ag presentation further. However, when using PBMCs as APCs, proliferation was observed only at the highest allergen concentration (100 μ g/ml), whereas no effects were seen at the concentrations that showed optimal complex formation (0.01–0.1 μ g/ml; Supplemental Fig. 3). These data suggest that for PBBCs, the complex formation is not the driving force of the initiation of the T cell response, and that their physiologic relevance for IgE–FAP may be limited. Although this seems in contrast with a vaccination study that showed that complement-containing IgG–influenza complexes can be presented by PBBCs, the data do fit with the data from a recent *in vivo* mouse study (1, 39). This study showed that CD23⁺ B cells do not present IgE–allergen complexes themselves but transport them to B cell follicles and transfer them there to CD11c⁺ cells. Depleting these CD11c⁺ cells abrogated the Ag presentation, indicating that these cells were essential for Ag presentation. Interestingly, a role for CR2⁺ B cells in the transport of immune complexes into follicles also has been described (40, 41).

In conclusion, allergen–Ab complexes formed in FA patients contain IgE, IgG1, and IgG4 Abs. Their levels influence the composition of the complexes and the subsequent binding to receptors on B cells: mixed IgE/IgG-containing complexes bind not only via CD23, but also via CR2. Preliminary data suggest that PBBCs are not directly involved in IgE–FAP. Moreover, considering the differences in the binding pattern of allergen–Ab complexes and in Ag presentation between EBV-B cells and PBBCs, it is questionable whether EBV-B cells can be used as a representative model for complex binding and IgE–FAP. Therefore, future studies should address the implications of the binding of mixed IgE/IgG-containing complexes to both CD23 and CR2 on Ag presentation and allergic responses *in vivo*.

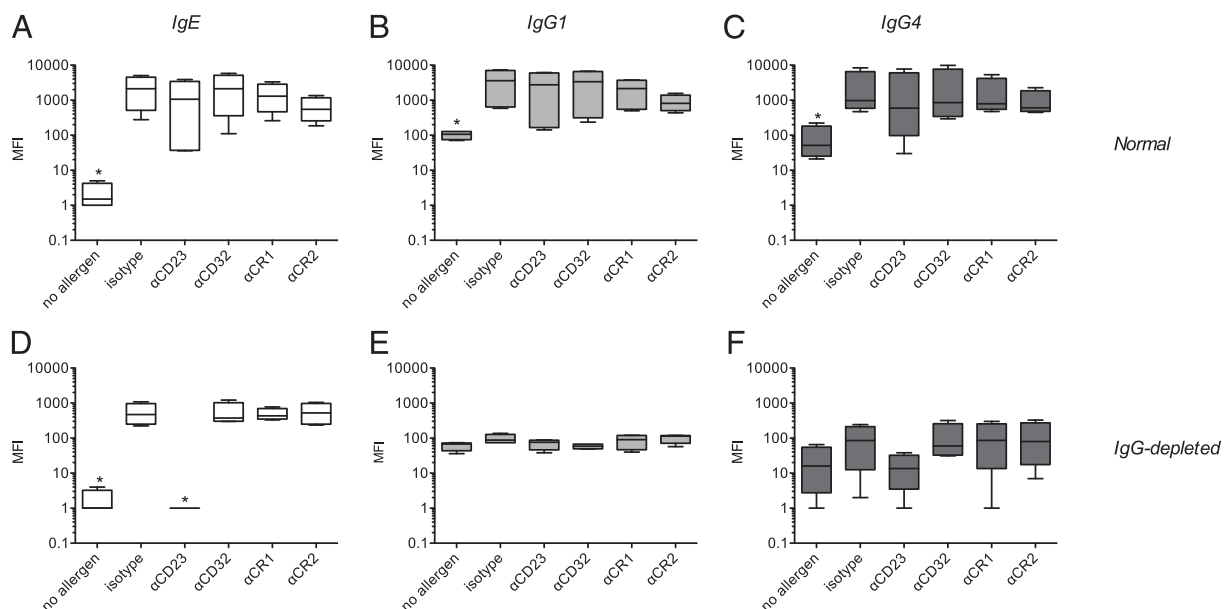


FIGURE 7. Effect of IgG depletion on receptor-mediated complex binding to PBBCs. MFI of IgE (**A**, **D**), IgG1 (**B**, **E**), and IgG4 (**C**, **F**) present in complexes binding to PBBCs with and without CD23, CD32, CR1, or CR2 blocking. Complexes were formed by incubating normal or IgG-depleted plasma from four PA patients with Ara h 2. All conditions were compared with isotype control with allergen. * $p < 0.05$.

Disclosures

The authors have no financial conflicts of interest.

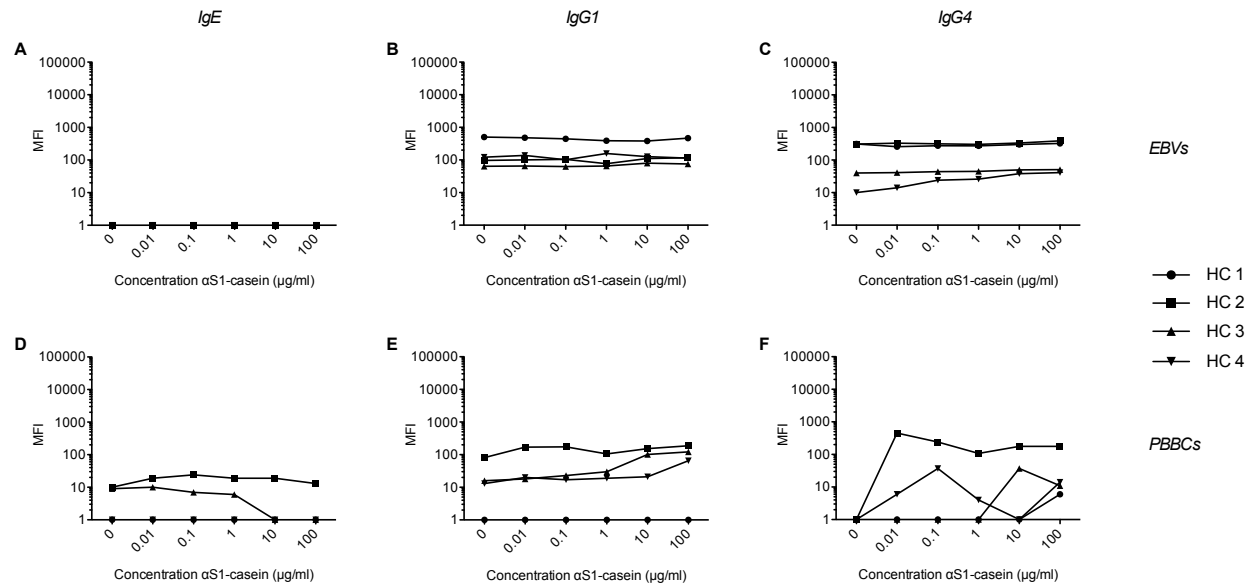
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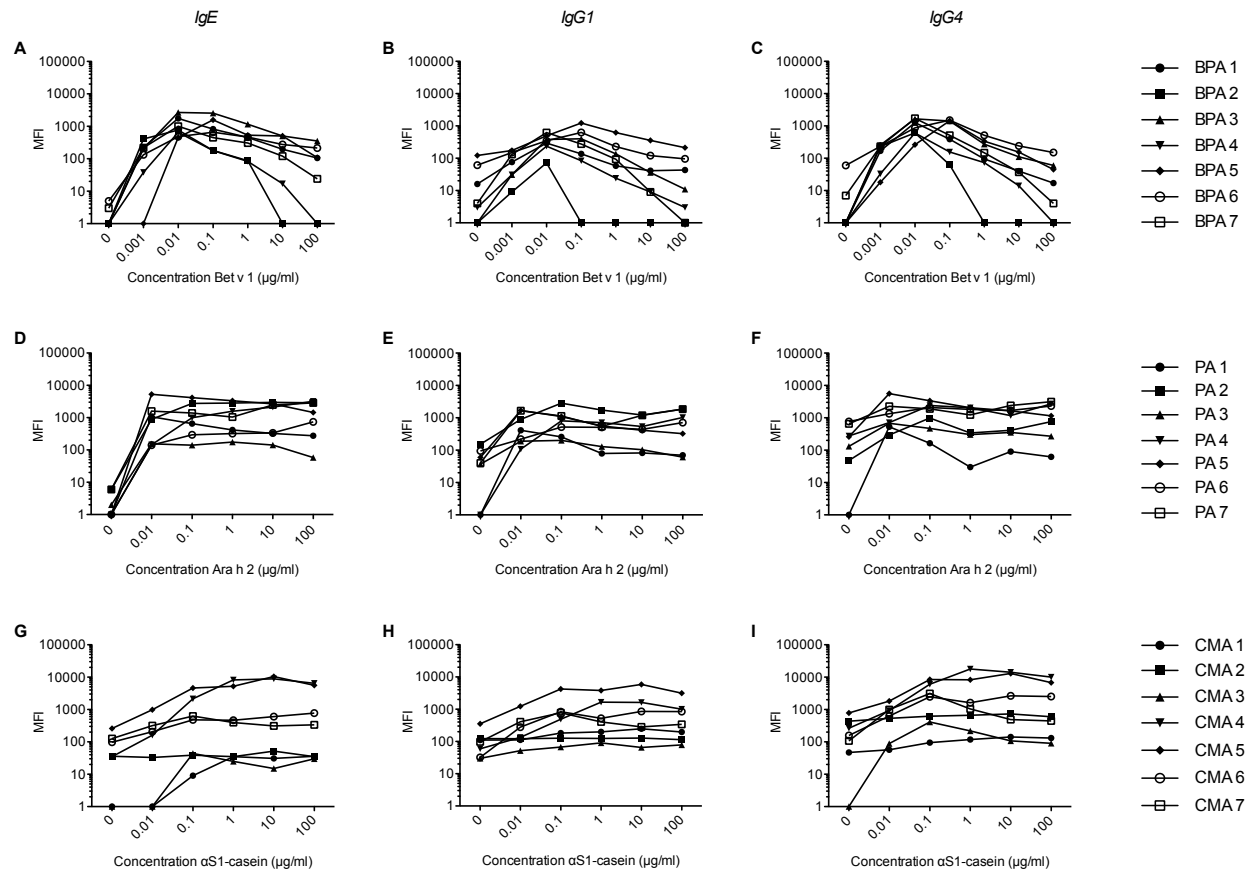
Supplemental Table I: Specific antibody levels to major allergens in patient samples used for *in vitro* complex formation.

Patients	Major allergen	Samples	IgE (AU)	IgG1 (AU)	IgG4 (AU)
HC 1	α S1-casein	Plasma	nd	703	310
HC 2	α S1-casein	Plasma	nd	632	475
HC 3	α S1-casein	Plasma	nd	233	334
HC 4	α S1-casein	Plasma	nd	407	1137
BPA 1	Bet v 1	Plasma	130	40	7
BPA 2	Bet v 1	Plasma	118	2	3
BPA 3	Bet v 1	Plasma	395	19	20
BPA 4	Bet v 1	Plasma	69	12	20
BPA 5	Bet v 1	Plasma	170	144	27
BPA 6	Bet v 1	Serum	218	59	98
BPA 7	Bet v 1	Serum	271	40	25
PA 1	Ara h 2	Plasma	575	102	15
PA 2	Ara h 2	Plasma	1483	795	66
PA 3	Ara h 2	Plasma	242	340	88
PA 4	Ara h 2	Plasma	922	915	46
PA 5	Ara h 2	Plasma	892	53	11
PA 6	Ara h 2	Plasma	373	383	974
PA 7	Ara h 2	Plasma	292	131	30
CMA 1	α S1-casein	Serum	107	146	344
CMA 2	α S1-casein	Plasma	405	147	22520
CMA 3	α S1-casein	Plasma	458	101	504
CMA 4	α S1-casein	Serum	16877	1598	3801
CMA 5	α S1-casein	Plasma	1547	703	721
CMA 6	α S1-casein	Plasma	302	632	1167
CMA 7	α S1-casein	Plasma	365	233	920

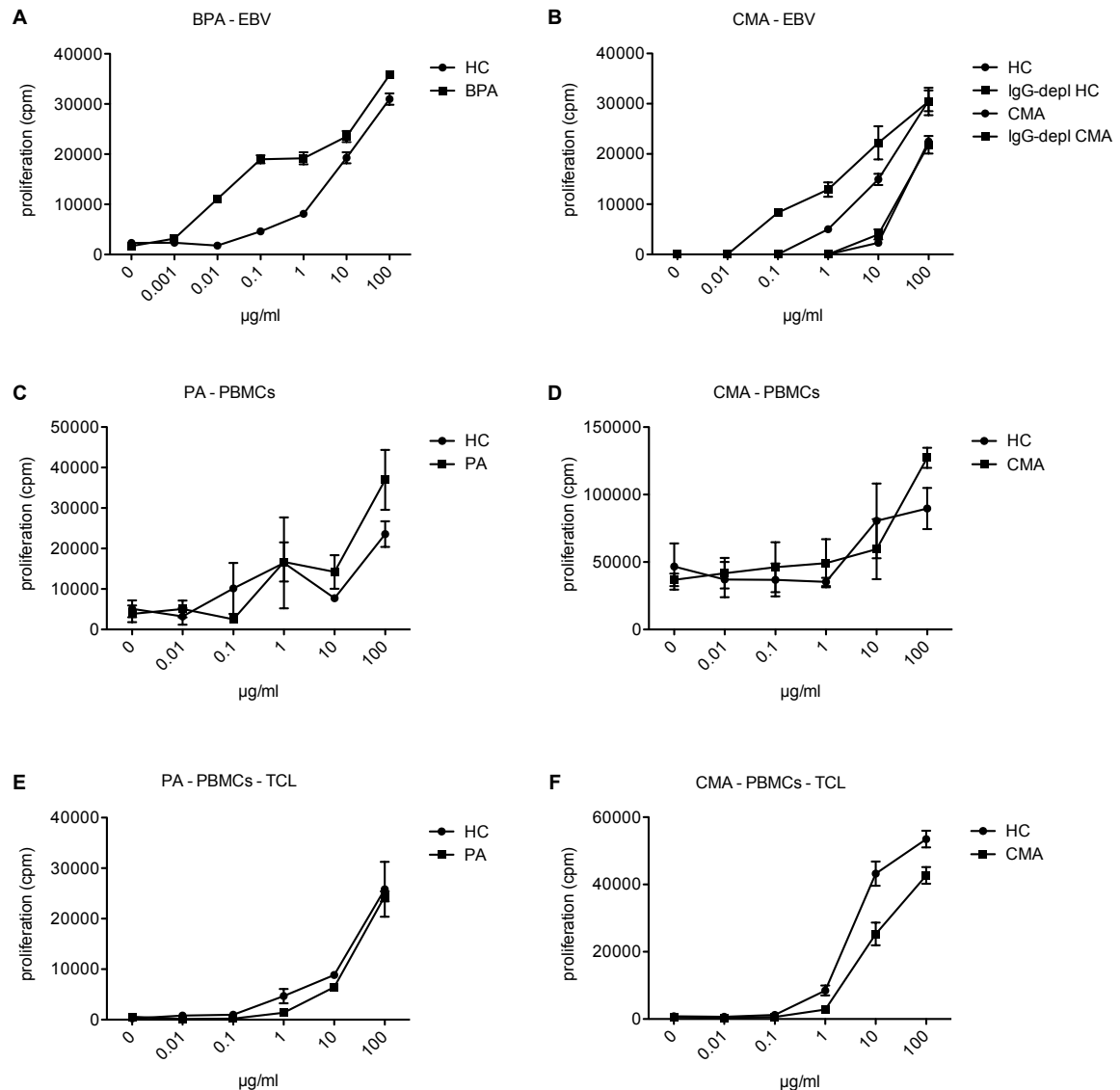
HC, healthy control; BPA, birch pollen allergy; PA, peanut allergy; CMA, cow's milk allergy; AU, arbitrary units; nd, not detectable.



Supplemental Figure 1: Allergen-antibody complex formation in healthy controls. MFI of IgE (A, D), IgG1 (B, E) and IgG4 (C, F) present in complexes binding to EBV-B cells (A, B, C) or PBBCs (D, E, F), after incubation of plasma samples of four healthy controls with different concentrations of the major allergen α S1-casein.



Supplemental Figure 2: Allergen-antibody complex binding to EBV-B cells. MFI of IgE (A, D, G), IgG1 (B, E, H) and IgG4 (C, F, I) present in complexes binding to EBV-B cells, after incubation of plasma/serum samples of seven BPA (A, B, C), PA (D, E, F) and CMA (G, H, I) patients with different concentrations of the major allergens Bet v 1, Ara h 2 and α S1-casein, respectively.



Supplemental Figure 3: Effect of complex binding on antigen presentation to T cells.

In general, complexes were formed as described in the article. For the experiments with the T cell clones/lines, the complexes were incubated for one hour with APCs before adding the T cells. Proliferation was determined by measuring [^3H]-thymidine incorporation.

(A) Proliferation of a BP-specific T cell clone, with its autologous EBV-B cells as APC (kind gift of P. Adler Würtzen, ALK Abellö). Proliferation is 100x more efficient upon complex binding using plasma from a BPA than from a HC subject.

(B) Proliferation of a CM-specific T cell clone, with its autologous EBV-B cells as APC. Proliferation is 10x more efficient upon complex binding using plasma from a CMA than from a HC subject. IgG-depletion of plasma has no effect when using HC plasma, but induces proliferation at a 10x lower allergen concentration when using CMA plasma.

(C,D) Proliferation of PBMCs from a PA or CMA subject in the presence of PA or CMA plasma, as compared to HC plasma. Only at the highest concentration, proliferation in presence of PA or CMA plasma exceeds proliferation in presence of HC plasma. This concentration is much higher than the optimal concentration for complex binding.

(E,F) Proliferation of a P- or CM-specific T cell line from a PA or CMA subject, with its autologous PBMCs as APC. Proliferation is not enhanced upon complex binding using PA or CMA plasma as compared to HC plasma.