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Research paper

Effect of IL-15 on IgG versus IgE antibody-secreting cells in vitro

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ABSTRACT

Immunoglobulin E (IgE) antibodies are major contributors to the pathology of atopic and allergic diseases as well as to immune response to helminth infections. Development of an adequate immunoglobulin G (IgG) immune response against infectious agents and vaccine antigens is considered in most cases as crucial for protection from disease. *In vivo* and *in vitro* production of IgE and IgG depends on cytokines and other soluble factors. Recently it has been shown that IgG antibody secreting cells (ASCs) can be generated by *in vitro* maturation of blood cells with Interleukin- (IL-) 15 and CpG DNA or other stimulation cocktails, while IgE-ASCs develop upon cultivation with anti-CD40 and IL-4.

In the present study we employed an enzyme linked immunospot assay (ELISPOT) to assess the capacity of individuals to develop into either IgE-ASCs or IgG-ASCs upon stimulation with different combinations of stimulation cocktails in order to investigate the influence of cytokines that are dysregulated in IgE-mediated immune reactions on ASC generation.

Furthermore, we modified the method to assess IgG- and IgE-ASCs specific for two model antigens causing allergic rhinitis in humans. We demonstrate that IL-15, which is important for development of IgG-ASCs, decreases the number of IgE-ASCs when added to media commonly used for *in vitro* development of IgE-ASCs. We show that our method is suitable for the detection of specific and non-specific IgE-ASCs and IgG-ASCs and allows the investigation of the interplay between IgG-ASCs and IgE-ASCs in different populations.

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1. Introduction

Antigen (Ag) specific memory is characterized by fast and efficient immune reaction upon repeated contact with the Ag and is mediated by antibodies (Ab), which are secreted from Ab-secreting cells (ASCs) or plasma blasts. *In vivo* these cells are known as long lived plasma cells and are terminally differentiated and homed in the spleen or bone marrow in certain survival niches (Manz et al., 2005). Memory B-cells persist in the periphery of humans and other

mammals and are able to differentiate into ASCs under different stimuli such as persistence of Ag, cytokines or other signals (Ochsenbein et al., 2000). These cells may cause protective immunity even when the amount of circulating Ab is low (Leyendeckers et al., 1999; Ahmed and Gray, 1996). Recently, it has been shown that memory B-cells develop into IgG secreting plasma cells in the presence of CpG oligonucleotides (CpG) and Interleukin- (IL-) 15 *in vitro*. Bernasconi et al. proposed that memory B-cells are constantly activated by polyclonal stimuli *in vivo* resulting in a constant level of plasma cells. This memory is theoretically available throughout the entire human lifespan (Bernasconi et al., 2002).

In order to characterize ASC a plaque formation assay was developed in 1963 by Jerne et al. and further improved by Czerkinsky et al. and Crotty et al. This enzyme-linked immunospot assay (ELISPOT) allows the enumeration of

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ASCs according to the Ag specificity and Ab class (Jerne and Nordin, 1963; Czerkinsky et al., 1983, 1988; Crotty et al., 2004).

We aimed to adjust this method to detect the intra-individual capability to develop IgE-ASCs as well as IgG-ASCs upon stimulation with different cytokines or stimuli. The ELISPOT technology was chosen because it can be performed in settings where technologies such as multicolor flowcytometers are not easy to access. In addition, ELISPOT can be conducted with a relatively low amount of blood. This is of particular importance when vulnerable populations are investigated, such as young children with parasitic diseases who often have a low hemoglobin level or suffer from comorbidity. Since the IgE ELISPOT was a commonly used method in the 1990s and is now replaced by other methods measuring cells directly, we first modified this technique for the detection of specific and non-specific IgE-ASCs and IgG-ASCs from *ex vivo* isolated peripheral blood mononuclear cells (PBMCs) in adult volunteers. To achieve this the culture medium was complemented with different cytokines proposed to have an impact on either IgE- or IgG-ASC development *in vitro*. Isotype switching to IgE-ASCs *in vitro* usually occurs upon stimulation of the cells with CD40 ligand followed by a signal of a specific cytokine, typically by IL-4 (Vallé et al., 1989). It has been shown that IL-21 has an influence on Ig production (Ozaki et al., 2002) and especially on IgE production *in vitro* (Caven et al., 2005). Recently it was shown that IL-21 favors the generation of IgE-ASCs *in vitro* (Kobayashi et al., 2009). However the exact role of each factor remains controversial (Suto et al., 2002).

Secondly, we investigated the relationship between *in vitro* generated IgE-ASCs and IgG-ASCs. More specifically, we examined whether cytokines that stimulate B-cells *in vitro* to develop into IgG-ASCs have a positive or negative effect on *in vitro* generation of IgE-ASCs.

2. Materials and methods

2.1. Study subjects

The study was conducted at the Institute of Tropical Medicine at the University of Tübingen, Germany. A total of 25 adults aged from 24 to 59 years (mean 32.4 years) were included into the study. The study was approved by the Ethics Committee of the Medical Faculty at the University Hospital Tübingen.

2.2. Measurement of total and allergen-specific IgE

Total serum IgE was assessed by ELISA technology. In brief ELISA Microtiter plates (NUNC™ Maxisorp) were coated with 1 µg anti human IgE (Sigma) diluted in phosphate buffered saline (PBS) (Gibco). After washing the plates 4 times with a washing buffer containing 0.5 M NaCl (Merck), 0.1% Tween 20 (Sigma) in PBS the plates are blocked with blocking buffer (3% non fat milk powder (Lasana), 0.1% Tween 20 in PBS) for 1 h. After another washing step serum samples diluted 1/100 in dilution buffer (1% non fat milk, 0.1% Tween 20 in PBS) were further serially diluted in duplicates with the same buffer. After 2 h of incubation followed by another washing step plates were incubated for 1 h with anti-human IgE-peroxydase (Sigma) diluted 1/3000 in dilution buffer.

After washing the plates were incubated with TMB ONE (KEM EN TEC) for 20 min. The reaction was stopped with 0.2 M H₂SO₄ (Merck 32%) and plates were analyzed at 450 nm (reference 620 nm) on a ELISA plate reader (Asys Expert 96, Type G018065). As standard, purified human polyclonal IgE (Acris) in PBS was employed.

To assess if the volunteers were positive for allergen specific serum IgE a screening assay (Polycheck®, BioCheck, Germany) was performed to screen the sera of all study participants for the presence of two major Ags causing allergic rhinitis in European individuals following the manufacturer's instructions. Briefly, 20 lines of inhalant allergens are readily coated together with 5 lines of calibrators on a carrier membrane located in the well of the Polycheck® chip. The serum of each volunteer was incubated for one hour to allow binding of allergen specific IgE. Non-bound serum components were removed by washing. To detect allergen-bound IgE, monoclonal ligand-labeled anti-IgE antibody was added as recommended and incubated again for 1 h followed by another wash step. A substrate solution was added to visualize specific immune complexes. The color intensity of the lines is thought to be proportional to the allergen specific IgE concentration in the serum of the volunteer. Interpretation of results was done using BioCheck Software (BIS).

2.3. Generation of peripheral blood mononuclear cells (PBMCs)

Peripheral venous blood was collected using Li-Heparin tubes (Sarstedt) and mixed 1:1 with HBSS (Life Technologies). PBMCs were separated by gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Thereafter, the PBMCs were washed twice with HBSS. PBMCs were further washed once with RPMI-1640 medium (Sigma-Aldrich) completed with sodium pyruvate, MEM non-essential amino acids, L-glutamine, penicillin-streptomycin solution, 2-mercapto-ethanol (all Life Technologies), and heat-inactivated fetal calf serum (FCS) (PAA) prior to cell culture.

2.4. Generation of ASCs

To generate ASCs, PBMCs prepared from peripheral blood were counted in a Neubauer counting chamber and diluted in complete medium at a final concentration of 1×10^6 PBMCs/ml and seeded to 24-well flat bottom culture plates (Corning Costar) at a final volume of 1 ml medium. For IgE-ASC development 50 ng/ml IL-4 (Immunotools) and 1 µg/ml anti-CD40 (R&D Systems) were added to the medium or supplemented additionally with 25 ng/ml IL-21 (Immunotools). For IgG-ASC development 2.5 µg/ml CpG-2006 (TIB-MOLBIOL) and 10 ng/ml recombinant human IL-15 (R&D Systems) were added to complete medium. To evaluate the impact of IL-15 and CpG on IgE-ASC development these stimuli were added to the medium as indicated in Table 1. The cells were incubated at 37 °C in 5%CO₂ for 6 days.

2.5. Enumeration of ASCs by ELISPOT

On day 5, ELISPOT plates (96-well PVDF membrane, Millipore) were coated overnight at 4 °C with either goat anti-human IgG-gamma chain specific antibodies (Sigma), for detection of total IgG-ASCs or anti-IgE (Sigma) for IgE-ASCs. For measurements of specific IgG-ASCs and IgE-ASCs plates were coated with either timothy grass pollen (*Phleum pratense*) or

Table 1

Different combinations of stimuli used in culture medium for the generation of ASCs.

Medium*	Stimulus
Medium I	anti-CD40, IL-4; IL-21
Medium II	anti-CD40; IL4; IL-21; CpG
Medium III	anti-CD40; IL-4; IL-21; IL-15
Medium IV	anti-CD40; IL-4
Medium V	CpG; IL-15
Medium VI	No stimulus

* RPMI-1640 medium supplemented with different combinations of stimuli.

birch tree pollen (*Betula verrucosa*) (both from Squarix Biotechnology, Germany). All plated Ags were diluted at 5 µg/ml in sterile PBS. To block unspecific binding on day 6, the coated plates were washed twice with PBS and blocked with complete medium for 1 h at 37 °C in 5% CO₂. Meanwhile cells were washed, counted and then seeded into the wells in duplicates. Serial dilutions were performed with a starting concentration of 2×10^4 cells/ml for the detection of IgG-ASCs and 2.5×10^5 cells/ml for IgE-ASCs.

During the incubation period at 37 °C in 5% CO₂ cells were allowed to secrete Abs (3 h for IgE and 2 h for IgG).

The plates were washed four times with PBS-0.1% Tween-20 (wash buffer) and twice with PBS alone to remove all unbound cells and debris. To reveal IgE-ASCs, plates were incubated for 2 h at room temperature (RT) with anti-human IgE peroxidase (Sigma) diluted 1/500 in PBS-3% BSA (dilution buffer). For the detection of IgG-ASCs in each experiment a biotin-labeled anti-human IgG (Sigma) diluted 1/500 in dilution buffer followed by the incubation with the extravidine peroxidase (Sigma) was employed. Alternatively, in some experiments IgE was detected using a biotin extravidine system that consists of a biotin labeled anti-IgE (KPL) followed by incubation with the extravidine peroxidase. After incubation, plates were again washed four times with wash buffer and twice with PBS alone, followed by 10 min of incubation in the dark with 3-amino-9-ethylcarbazole (AEC) substrate (Sigma) diluted in NN-dimethylformamide and 0.1 M Sodiumacetate (Sigma) supplemented with 30% H₂O₂ (Sigma) followed by two filtrations (0.45 µm and 0.22 µm) to visualize the spots. In some experiments an AEC substrate kit (Sigma) was used. Plates were rinsed with water and dried at room temperature overnight.

2.6. Enumeration and documentation of spots

All ELISPOT plates were scanned using the CTL Immunospot ELISPOT reader and ASCs were counted using the CTL ELISPOT software (Cellular Technology Ltd), and confirmed by visual inspection, as described in Blanchard Rohner et al. (2008). IgE-ASCs were smaller in size and less intense in color (when assay was performed with anti-human IgE peroxidase) and therefore counted using ImageJ (ImageJ 1.42v, NIH, USA).

The frequency of ASCs is expressed as cells per 1×10^5 PBMCs.

2.7. Statistical analysis

Stata (version 9.1, StataCorp, USA) was used for the statistical analysis. Ag-specific memory B-cell frequencies were summarized using medians with 5th–95th percentile. ASC

frequencies below the sensitivity of the ELISPOT were arbitrarily set to 0. A positive response was defined as the presence of at least 4 Ag-specific spots in the total of all replicate wells.

Because of the small sample size of this study, no multivariate analysis was performed. For all statistical analyses, two-sided p values less than 0.05 were considered statistically significant.

3. Results

3.1. Study subjects and detection of allergen specific and total serum IgE

The primary goal of the study was to establish a viable method to measure the capacity of an individual to develop in vitro generated total and Ag specific IgE-ASCs as well as IgG-ASCs. To detect Ag specific IgE-ASCs we employed two major Ags triggering allergic rhinitis in European adults as model Ags. To ensure that enough individuals display positive serum IgE levels for these model antigens we included healthy donors as well as individuals reporting allergic disease, preferentially allergic rhinitis.

A total of 25 adults aged from 24 to 59 years (mean 32.4 years) participated in the present study, twelve of them reported allergic disease.

In order to detect Ag specific ASCs by ELISPOT assay, we first assessed if specific IgE against the two selected model antigens was measurable in sera of the study participants using an immunoassay (Polycheck®, BioCheck).

Sera of 24 volunteers were screened for IgE antibodies against timothy grass pollen and/or birch pollen, the selected model Ags for the ELISPOT assay and additionally for total serum IgE. Fig. 1 shows representative results of a volunteer negative for the screened Ags and of a volunteer positive for both antigens (bands are indicated). In total, 10 of all screened volunteers had detectable IgE levels against either timothy grass pollen or birch pollen. Eight were positive for both allergens and only two for one of the two antigens. Fourteen individuals were serum negative. Four volunteers were completely negative for total serum IgE (data not shown).

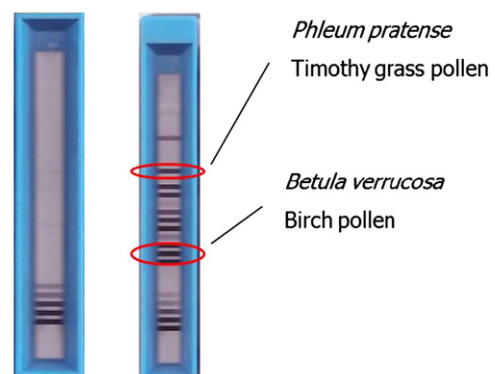


Fig. 1. Sera of 24 volunteers were examined with the Polycheck® screening technology for the detection of allergen specific serum IgE. The left strip shows a typical result of a volunteer with no history of allergic disorder and the right strip represents a typical result of another donor tested positive for serum IgE against birch pollen and timothy grass pollen (as indicated in red) and other allergens. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Detection of ASCs by ELISPOT

To establish a stable and reliable protocol to assess the variation between each individual to develop in vitro IgE-ASCs and IgG-ASCs we employed and modified the ELISPOT technology and investigated the influence of different cytokines on IgE-ASC synthesis of cultured PBMCs. In total, 6 media with different cytokine cocktails were investigated to determine their influence on IgE- as well as on IgG-ASC development.

It was noticeable that IgE specific spots were smaller in size and less intense in color compared to IgG-ASCs when incubated with the IgE peroxidase Ab. However when experiments were performed with a biotin conjugated antibody and extravidine peroxidase as performed in the IgG ELISPOT assay the spots had a similar size and color as the IgG spots without a major increase in background noise (Fig. 2).

3.3. Influence of IL-15, CpG and IL-21 on detection of IgE-ASCs by ELISPOT

To assess if IL-15 or CpG, which are able to influence IgG-ASCs maturation in vitro, have an effect on the generation of IgE-ASCs, we added these stimuli to the cell cultures incubated with cytokine cocktails appropriate for IgE synthesis. Since it was recently

reported that addition of IL-21 to medium containing anti CD40 and IL-4 enhances the development of IgE-ASCs in vitro we expected medium I (supplemented with anti CD40, IL-4 and IL-21) to be superior compared to medium IV (anti-CD40 and IL-4 alone). We added either IL-15 or CpG to medium I to assess the effect of IL-15 and CpG on the “most effective” medium for the generation of IgE-ASCs. IL-15 added to medium I significantly decreased the number of detectable IgE-ASCs. The same trend was seen by the addition of CpG to medium I although it was not statistically significant. Fig. 3 shows the results of IgE-ASC and IgG-ASC number detected by ELISPOT upon stimulation with different media given in Table 1.

3.4. Allergen-specific IgE-ASCs assessed by ELISPOT

PBMCs from volunteers either positive for one or both of the Ags were cultured with all of the supplemented media and tested for their ability to secrete Ag specific IgE.

The results are represented in Fig. 4. There was no association between total and specific serum IgE levels and the number of IgE-ASCs in this IgE-positive population. As shown in former experiments the addition of IL-15 to the IgE-culture medium I reduced the number of specific IgE-ASCs. The negative influence of CpG was not significant (see Fig. 4).

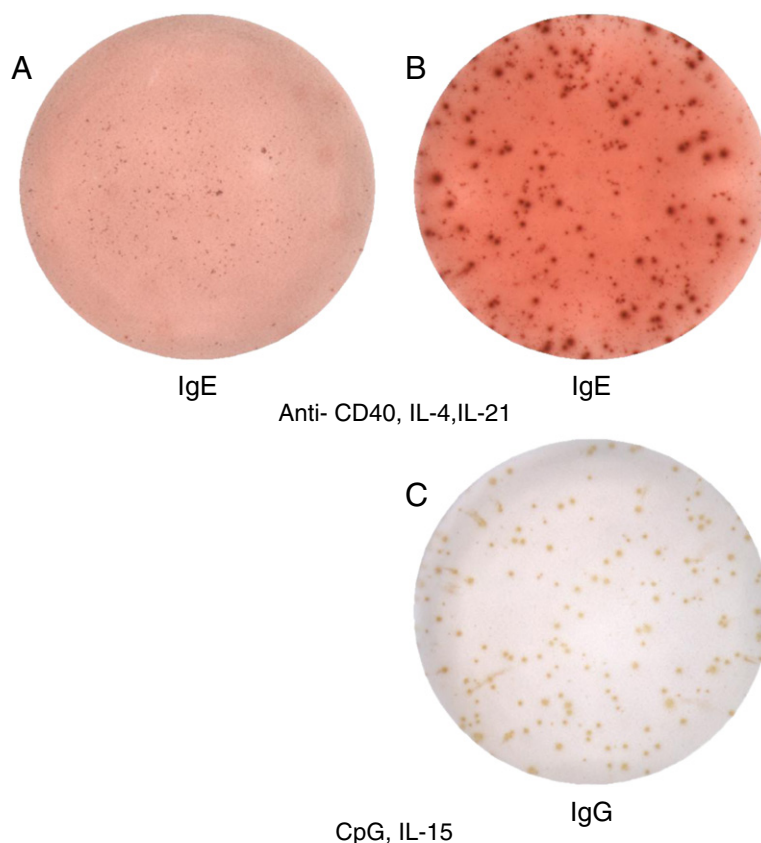


Fig. 2. Typical results showing Elispots of IgE-ASCs upon stimulation of PBMCs for 6 days with a combination of anti-CD40, IL-4 and IL-21. The size of the spots is smaller and the spots are less intense in color when ELISPOT assay was performed with the IgE-peroxydase antibody (A). For some experiments a biotin conjugated IgE antibody was used resulting in darker and larger spots (B) comparable with the spots detected from IgG-ASCs (C). The figure shows typical representative results of different experiments.

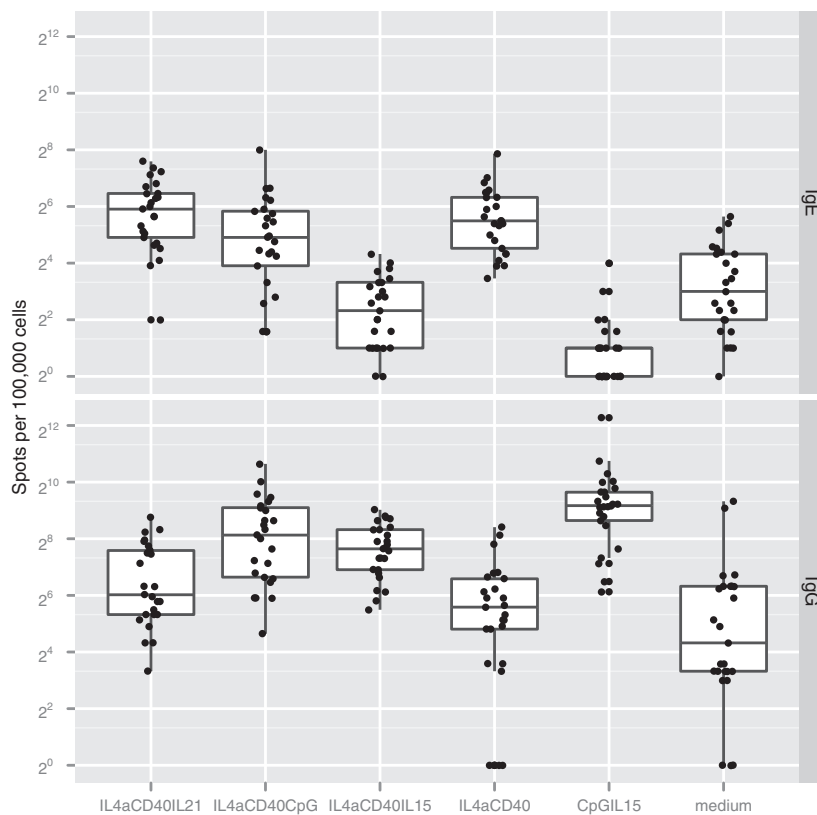


Fig. 3. A combination of anti-CD40, IL-4 with or without IL-21 represents a suitable stimulus for the generation of IgE-ASCs (upper panel) and the combination of IL-15 plus CpG for generation of IgG-ASCs (lower panel). Addition of IL-15 to medium 1 decreases the number of detectable IgE-ASCs (upper panel). Shown are boxplots and individual measurements as dots. Boxplots represent the number of spots per 1×10^5 cells.

4. Discussion

Elevated levels of serum IgE are common in allergic disorders as well as in individuals infected with parasites especially when they experienced frequent infections since early childhood on (Yazdanbakhsh et al., 2002; Rodrigues et al., 2008). There is evidence for persistent IgE-secreting plasma cells because specific IgE persists for several years even following therapy and absence of re-exposure (Mitre and Nutman, 2006).

In the present study we adapted the ELISPOT technique previously used in frequent studies in the nineties. Unfortunately, most of the protocols do not mention available Ab sources as well as exact concentrations of Abs and cytokines and therefore a new protocol needed to be established.

To assess IgG-ASCs, PBMCs were cultured for several days with the combination of CpG and IL-15 as suggested by Bernasconi et al. (2002). This combination of stimuli was favored during our recent experiments when establishing the ELISPOT assay for vaccine specific ASCs during a phase I trial investigating the safety and immunogenicity of GM22 a new malaria vaccine candidate (Esen et al., 2009; Mordmüller et al., 2010), because CpG and IL-15 are recombinant and readily available in large quantities and therefore suitable for clinical trials in contrast to ingredients for alternative stimulation cocktails used for in vitro ASC development. In order to generate IgE-ASCs, we employed IL-4 and anti-CD40 as suggested by Jabara et al. (1990) and additionally we added IL-21 to the

“classic” medium as this has been reported to enhance IgE production (Caven et al., 2005; Caven et al., 2007; Kobayashi et al., 2009). Furthermore, we investigated whether IL-15 or CpG as effective inducers for IgG-ASCs influence the generation of IgE-ASCs. During the course of experiments we found that these two agents suppressed the generation of IgE-ASCs. IL-15 had a particularly strong suppressive effect. So far only a small number of reports investigated the influence of IL-15 and CpG on the development of IgE mediated immune reactions. One study showed that IL-15 prevents allergic rhinitis in mice (Aoi et al., 2006). In another study also conducted with mice, it was shown that IL-15 blockade resulted in induction of allergen specific T-cells and inflammation (Rückert et al., 2005). Association of an IL-15 polymorphism with asthmatic disorders has also been reported (Bierbaum et al., 2006). An association of low IL-15 levels with increased IgE levels has been suggested recently as PBMCs isolated from volunteers suffering from atopic dermatitis secreted a lower amount of IL-15 upon unspecific stimulation with staphylococcal enterotoxin B compared to healthy individuals. In this study the authors additionally measured an association of IL-15 on IgE and IgG by ELISA (Ong et al., 2002).

In the present study we show that the capacity of cells from different donors to develop into either IgE- or IgG-ASCs is strongly influenced by IL-15. Our findings support the idea that IL-15 as well as CpG could serve as therapeutic agents for treatment of allergic disorders as proposed by

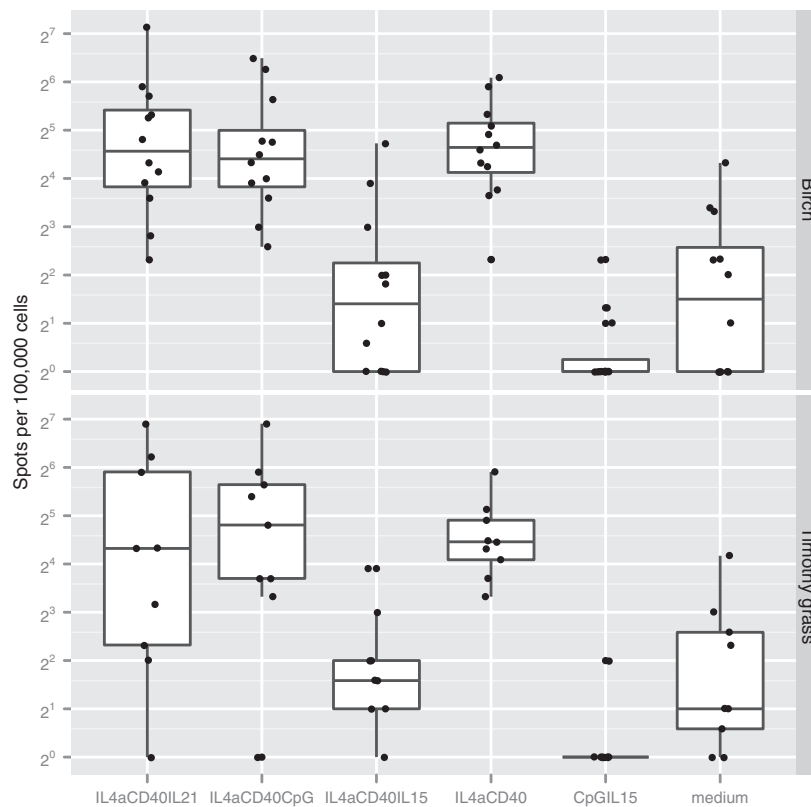


Fig. 4. Allergen-specific responses determined by ELISPOT 6 days after stimulation of PBMCs. ELISPOT plates were coated with birch pollen (upper panel) or timothy grass pollen (lower panel). Shown are boxplots and individual measurements as dots. The boxplots represent the number of spots per 1×10^5 cells.

Ishimitsu et al. (2001) and Kline (2007). The hypothesis of these authors is based on mouse experiments. In the present study, we confirmed these findings by *ex vivo* investigations in humans. Furthermore we show that IL-15 increases the number of IgE-ASCs when added to the “IgE medium”. This suggests that IL-15 could influence *in vivo* the generation of Ag specific IgG, for instance, during vaccination.

Since we assumed that the addition of IL-21 increases the IgE-ASC number and our primary objective was to establish a viable test for further clinical studies to examine the interplay of IgE-ASCs and IgG-ASCs in different populations, we added IL-15 and CpG to our assumed “most stimulating medium” and did not examine the effect of IL-15 and/or CpG in culture medium supplemented with anti-CD40 and IL-4 alone. In pilot experiments however we observed the same results. If more subtle effects are present has to be investigated in further studies with more individuals. However in the present study we have not identified the mechanism responsible for the decreased number of IgE-ASCs induced by IL-15. Furthermore we have not focused on the analysis if IL-15 might have a different influence on either healthy or allergic individuals resulting in a different pattern of IgE- and IgG-ASCs upon *in vitro* stimulation of the isolated PBMCs because our information was only based on reported medical history and screening of serum total IgE and serum IgE against common allergens. We have not done further examinations such as Ag challenge to verify and stage the reported allergies. This approach was selected because we intended to establish a

reliable protocol to assess Ag specific IgE-ASCs and therefore we chose two model Ags for allergic rhinitis, one of the most frequent allergies in Germany. In our setting we could not detect an association between total and specific serum IgE and the number of IgE-ASCs. To substantiate these findings further studies using much better defined participants need to be done, e.g. clinically verified and staged allergic versus non-allergic individuals or worm infected versus non-infected subjects.

In conclusion, we modified the ELISPOT technique to detect total and specific IgE-ASCs. These IgE-ASCs were compared to IgG-ASCs of lower frequency and less intense in color when the assays were performed with the peroxidase conjugated anti human IgE. First we thought that this might be due to a lower secretion rate of IgE-ASCs. However, this explanation seemed to be unlikely since it has been published that IgE-secreting cells exhibit the same individual secretion rates as IgG secreting cells (Werner-Favre et al., 1993). Therefore in some later experiments we intended to see if the spots resulting from IgE-ASCs became the same size and color intensity as the spots of the IgG-ASCs performing the assay with a biotin conjugated antibody as used for the IgG ASC detection. However we could not repeat the whole study with this approach. Nevertheless as well in these experiments it was obvious that the addition of IL-15 to medium I clearly decreases the number of detectable IgE-ASCs. Since IL-15 has an impact on the development of immunity against parasites, e.g. against *Toxoplasma gondii*

(Badra et al., 2010) we intend to investigate the influence of IL-15 on IgE- and IgG-ASCs of individuals with parasite infections and therefore with high serum IgE levels. Since ELISPOT requires only small amounts of blood and expensive or sensitive equipment is not needed, it is well suited for such a study in African children, a population most vulnerable to helminth infections.

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