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Allergen extract-induced interleukin-10 in human memory B cells inhibits immunoglobulin E production

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Summary

Background Elevated specific IgE antibody levels are common in atopic individuals, caused by T-helper type 2-dominated B cell activation. The induction of antigen-specific IL-10 secreting T cells is discussed as an important mechanism during specific immunotherapy. By contrast the presence and function of B cell-derived IL-10 is not well defined yet.

Objective We investigated whether type-I allergen extracts induce IL-10 expression in human B cells and analysed its functional role on IgE production.

Methods Human peripheral B cells were stimulated with grass pollen, house dust mite (HDM) (*Dermatophagoides pteronyssinus*; Der p) and dog allergen extract. Expression of IL-10 by activated human B cells was determined by flow cytometric analysis and ELISA. Functional analysis considering immunoglobulin production was assayed by ELISA.

Results The allergen extracts studied induced IL-10 expression in B cells. However, the ability to induce IL-10 differed between the allergen extracts. The most potent allergen extract was dog (169 ± 28 pg/mL), followed by grass pollen (141 ± 10 pg/mL) and HDM allergen (125 ± 11 pg/mL). Upon allergen extract stimulation only CD27 expressing memory B cells produced IL-10 and co-expressed the *very early activation antigen* CD69. The addition of allergen extracts to B cells activated by anti-CD40 and IL-4 selectively inhibited IgE which was dependent on allergen extract-induced IL-10. By contrast the other immunoglobulin subclasses like IgA, IgG or IgM were not altered upon allergen extract challenge.

Conclusion Our data indicate that allergen-activated memory B cells can modulate IgE production through secretion of IL-10.

Keywords allergen extracts, B cells, IgE, IL-10

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Introduction

Atopic diseases are characterized by elevated serum levels of IgE resulting from T-helper type 2 (Th2)-dominated B cell activation [1–3]. These diseases remain chronic and not curable, although specific immunotherapy, the only causal treatment available so far, is becoming more efficient. Nevertheless, the mechanisms underlying specific immunotherapy are not fully understood. Recent evidence suggest that IL-10 secreting and Foxp3⁺ regulatory CD4⁺ T cells, generated during specific immunotherapy or naturally by repeated high-dose antigen application, e.g. bee stings, mediate an IgG4-dominated B cell response and tolerance towards allergens [2, 4–9]. T regulatory cells

participate in suppression of antigen-presenting cells which support the generation of effector Th2 and Th1 cells, inhibit the induction of allergen-specific IgE and induce IgG4 and/or IgA [10]. The capacity of B cells to produce IL-10 upon direct allergen contact and its function has not been investigated yet. Human B cells produce a variety of different cytokines including IL-10, which is regulated by the B cell receptor (BCR) and/or CD40, IL-4 [11] and Toll-like receptor (TLR-9) [12]. IL-10 shows different effects on B cells, e.g. IL-10 promotion of plasmablast development and production of IgG [13, 14], IgA [15], IgM [16] and inhibition of IgE production [17, 18], antigen presentation, migration and production of pro-inflammatory cytokines such as IL-6 [16]. A limited number of structurally different proteins are frequent sensitizers of type-I allergies, such as pollen, or microbial

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enzymes, but their function on B cell activation remains to be elucidated.

Here we investigated the impact of different allergen extracts on IL-10 expression in human B cells. We selected three allergens according to their average frequency to cause of type-I sensitization, like grass pollen as common antigen, followed by house dust mite (HDM) allergen and the rather rare dog hair allergen. Our data show that all these allergen extracts have the potency to induce IL-10 expression in purified peripheral B cells, in negative correlation to their average frequency of type-I sensitization. For the first time, these cells were detected by flow cytometric analyses and our data show that IL-10 expression is restricted to the CD27⁺ memory B cell subset. The functional consequence of allergen extract-induced IL-10 was shown by a reduction of anti-CD40 and IL-4 driven IgE production in B cells. The production of other immunoglobulin subclasses was not altered. Our data show that allergen extract activated memory B cells produce and perceive IL-10, suggesting a novel immunomodulatory mechanism of specific immunotherapy on B cells, as well as a protective regulatory role on type-I sensitization.

Materials and methods

Cell preparation and cell culture

The study was approved by the local ethics committee and the informed consent was signed by each donor. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by density gradient centrifugation (Biochrom KG, Berlin, Germany). CD19⁺ B cells were positively selected to >99% purity by magnetic cell sorting (Miltenyi Biotec, Bergisch-Redbach, Germany) with CD19 Multisort beads as described before [19].

Naïve and memory B cells were sorted by fluorescence-activated cell sorting (FACS Aria cell sorter and FACSDiva software, both BD Biosciences, Freiburg, Germany) according to IgM and CD27 expression, after magnetic enrichment using CD19 beads. Populations were defined by the expression of IgM⁺CD27⁻ as naïve and IgM⁺CD27⁺ as memory B cells. The purity of the sorted B cell subsets exceeded 99%.

3 × 10⁶/mL total, naïve and memory B cells were cultured in flat-bottomed 48-well plates in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) (all from Biochrom KG). All cell cultures were carried out at 37 °C in humidified air and 5% CO₂.

Reagents

Anti-CD40 monoclonal antibodies (mAbs; 1 µg/mL), neutralizing anti-IL-10 mAbs (5 µg/mL; both R&D, Wiesbaden,

USA), recombinant human IL-4 (5 ng/mL, Immunotools, Friesoythe, Germany), CpG2006-oligonucleotide (3 µg/mL, TibMolbiol, Berlin, Germany) and goat-anti-human IgA/IgG/IgM-F(ab')₂ fragments (2 µg/mL, Jackson ImmunoResearch, Hamburg, Germany), lipopolysaccharide (LPS) (200 ng/mL, *E. coli* serotype 055:B5; both Sigma, Dreieich, Germany) were obtained commercially. Purified dog hair (Can.fl.), grass pollen (Phl.p5) and HDM allergen (*Dermaphagoides pteronissimus* 1, Der p) were from ALK-Scherax, Wedel, Germany (all were shipped lyophilized and reconstituted in complete cell culture medium) and used at 10 000 SQ; LPS concentration in the preparation: dog allergen < 165 pg/mL; grass pollen < 22.2 pg/mL; Der p 1 < 18.5 pg/mL. Antibodies used in flow cytometry were fluorochrome coupled anti-CD19 and anti-IL-10 (Miltenyi Biotec), anti-CD23 (Immunotech, Germany), anti-CD27, anti-CD38, anti-CD69 (all from BD Biosciences), anti-IgM (Dako, Hamburg, Germany). Dead cells were excluded using 1 µg/mL propidium iodide staining (Sigma). Flow cytometric analysis was performed using FACScalibur (BD Biosciences) and FCS express V2 software (DeNovo, Los Angeles, California).

Detection of interleukin-10 producing B cells

CD19⁺ B cells were cultured for 48 h with anti-CD40 and IL-4 with or without allergen extracts, and subsequently stimulated with 10 ng/mL polymethyl acrylate (PMA) and 1 µg/mL ionomycin (both Sigma) for 2 h. B cells were labelled with a high-affinity bivalent antibody matrix against CD45 (leucocyte common antigen [20]) and IL-10 (Miltenyi Biotec), which captures secreted IL-10 on the surface of the secreting cell. The cytokine secretion of the cells was performed by incubation under gentle rotation at 37 °C for 1 h. As negative control for this step served cells kept on ice during that time (data not shown). IL-10 is detected with a second fluorescent IL-10-specific antibody [21]. Finally, the cells were stained on ice using fluorescent coupled antibodies and analysed by flow cytometry under exclusion of dead cells using propidium iodide. Also, intracellular staining of IL-10 expressing B cells was also performed. Therefore, 3-day-cultured B cells were restimulated for 4 h with 10 ng/mL PMA and 1 µg/mL ionomycin including 2 h block with 2 µg/mL brefeldin A (all from Sigma) and fixation using 1% formaldehyde for 10 min.

Enzyme-linked immunosorbent assays

IL-10 and IgM was determined in the cell-free supernatants of B cells after 72 h of stimulation with and without allergen extracts (10 000 SQ) using cytokine-specific ELISA according to the manufacturer's instruction (R&D Systems, Wiesbaden, Germany). Immunoglobulin production was quantified from supernatants of B cells cultured for 10 days with and without anti-CD40 and IL-4 in the absence or

presence of 10 000 SQ allergen extract. IgE-levels were determined as previously described [19] using two different matched anti-IgE mAb clone HP6061 (Southern Biotech, Birmingham, Alabama, USA) coated on Maxisorb plates (Nunc, Weisbaden, Germany) and biotinylated anti-IgE mAb clone HP6029 (both Southern Biotech, Birmingham, Germany) detected. As enzyme streptavidin-alkaline phosphatase (Zymed, Muenchen, Germany) was used. The enzymatic activity was measured by the colorimetric analysis of *p*-nitrophenylphosphate (Sigma) at 405 nm in a plate-reader system (Dinotech Technology Incorporated, Edgewood, NY, USA). Detection of IgA, IgG and IgM were performed with matched antibody pairs (Jackson ImmunoResearch, Dianova, Hamburg, Germany) whereas the detection antibody was coupled to alkaline phosphatase and detected by cleavage of *p*-nitrophenylphosphate.

Statistics

All statistic calculations were performed using SPSS 12.0 software (SPSS Inc., Chicago, Illinois, USA). Statistical significant differences obtained by the non-parametrical Mann–Whitney *U*-test were considered at *P* values < 0.05.

Results

Allergen extracts induce interleukin-10 in human B cells

The potential of structurally different allergens, such as dog hair allergen (Can.fl), grass pollen (Phl.p5) or HDM (Der p 1) allergen to induce IL-10 expression in human B cells was investigated by adding them directly to highly purified CD19⁺ B cells *in vitro*. Time-course analysis revealed the maximum of B cell-derived IL-10 expression after 72 h, irrespective of the allergen extract used (data not shown). Titration identified a dose-dependent induction of IL-10 protein with an optimal concentration of 10 000 SQ of each allergen extract (correlating to 0.8 µg/mL Can.fl., 2.02 µg/mL Phl.p5 and 0.98 µg/mL Der p 1). The most potent allergen extract to induce IL-10 expression in human B cells was dog hair (210 ± 36 pg/mL), followed by grass pollen (202 ± 94 pg/mL) and finally HDM allergen Der p 1 (182 ± 57 pg/mL), as shown in Fig. 1. Triggering of TLR-9, which is highly expressed in memory B cells [22] contained in the B cell preparation (approximately 25%), by CpG2006 resulted in 231.5 ± 33.8 pg/mL IL-10 production. In order to determine whether the allergen extract-induced IL-10 production is specific or consequence of unspecific B cell activation, IgM was quantified from the supernatants after 3 days culture. Data show that the concentration of IgM was independent of allergen extract stimulation (data not shown).

Additionally, we investigated whether LPS contamination of the allergen extracts may contribute to the observed IL-10 expression in the supernatants, which could

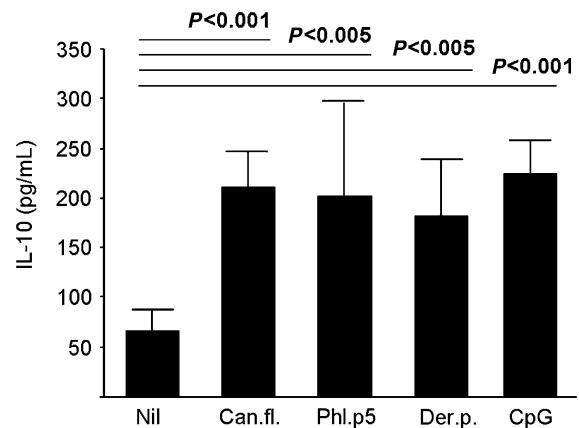


Fig. 1. Allergen extracts induce IL-10 expression in human B cells. Peripheral human B cells (>99% CD19⁺) were cultured for 72 h in the presence 10 000 SQ dog allergen (Can.fl.), grass pollen (Phl.p5), house dust mite (Der. p.) or CpG2006 (3 µg/mL). IL-10 expression was quantified from the cell free supernatants by ELISA. Data show arithmetic mean ± SEM from six individual experiments.

be derived from non-B cells (<1%) in the B cell preparation. Human B cells lack TLR-4 [23] and should not respond to LPS. However, to ensure these previously published data in our system we cultured B cells for 72 h with LPS (2 ng/mL) and measured IL-10 consecutively (data not shown). In this setting we were not able to detect any significant IL-10 in comparison with unstimulated cells. Moreover, we performed an IL-10 secretion assay with B cells stimulated with LPS. The percentage of IL-10 producing B cells was the same as within unstimulated cells (see Fig. 2a).

Allergen extracts induce interleukin-10 expression in memory, but not naïve CD19⁺ B cells

Flow cytometric analysis was performed in order to determine the B cell subset secreting IL-10 upon allergen extract stimulation. CD19⁺ human B cells were cultured for 48 h in the presence of dog allergen Can.fl., anti-CD40 and IL-4 (data not shown) or antigen-receptor cross linking F(ab)₂ fragments, or CpG2006 triggering TLR-9, followed by a subsequent 2.5 h PMA and ionomycin stimulation. The secreted surface-bound IL-10 was detected by flow cytometry (Fig. 2). The specificity was controlled by intracellular cytokine staining (data not shown).

Upon stimulation with allergen extract alone, IL-10 production was exclusively induced in CD27⁺ memory B cells (7.2%) in comparison with unstimulated cells (0.6%), as shown in Fig. 2b. This finding was confirmed by analysis of naïve (IgM⁺CD27⁻) and memory (IgM⁺CD27⁺) B cells sorted before the cell culture (Fig. 3). IL-10 producing B cells stimulated with allergen extract alone co-expressed the *very early activation antigen* CD69, but not the CD23 (Fig. 2b). These data show that upon allergen extract stimulation a distinct memory B cell subset is responsible for IL-10 production.

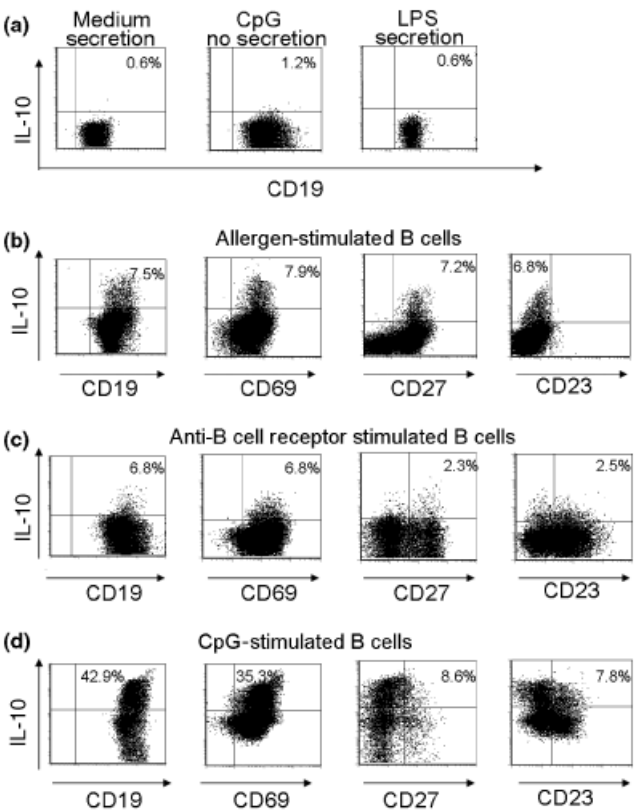


Fig. 2. Phenotype of allergen extract-induced IL-10 producing B cells. Human B cells were cultured in the presence of (a) medium or lipopolysaccharide (LPS) (200 ng/mL), (b) allergen extracts (10 000 SQ), (c) anti-B cell receptor crosslinking F(ab)₂' fragments (2 µg/mL) or (d) CpG2006 (3 µg/mL) for 3 days, followed by a 2 h polymethyl acrylate (PMA)/ionomycin restimulation. The cells were labelled with an IL-10 capturing matrix and incubated for 1 h at 37 °C for cytokine secretion, and stained with a second, fluorochrome-conjugated anti-IL-10 antibody. Expression of the surface molecules CD19, CD27, CD23, CD69 and secreted IL-10 was detected by flow-cytometry. Dot blots are gated on viable B cells. Data show a representative out of three identical experiments.

Allergen extract-induced interleukin-10 expression in B cells signals through B cell receptor and Toll-like receptor-9

By triggering the BCR and TLR-9 independently and together with allergen extract stimulation, we sought to determine possible signalling pathways involved in allergen extract-induced IL-10 expression. Activation of the BCR by addition of a saturating concentration anti-BCR-F(ab)₂' fragments induced IL-10 expression in human B cells, and was further increased by maximal triggering of TLR-9 by +263%±2.8 (Fig. 4). Stimulation of CD19⁺ B cells with allergen extracts and additional BCR cross linking, or with CpG2006, in the presence of allergen extracts augmented IL-10 expression by +57%±0.5 and +36.3%±2.4, respectively. As BCR and TLR-9 triggered IL-10 expression is increased upon addition of allergen extracts, the data indicate that the signalling is not exclusive for BCR or TLR-9.

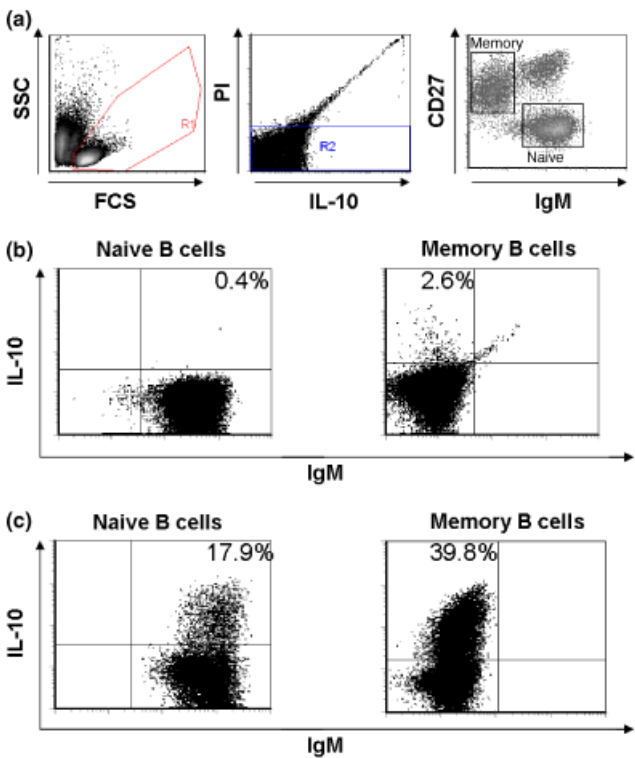


Fig. 3. Allergen extract-induced IL-10 secretion of naïve and memory B cells sorted before cell culture. (a) Sorting strategy of live CD19⁺ B cells according to CD27 and IgM surface expression. Sorted naïve and memory B cells were stimulated for 72 h with (b) allergens (10 000 SQ) or (c) CpG2006 (3 µg/mL), followed by a 2 h polymethyl acrylate/ionomycin restimulation. IL-10 secreting B cells were detected as described in Fig. 2. The surface expression of CD27, IgM and IL-10 was determined by flow cytometry. The dot blots are gated on live lymphocytes and data show one representative out of three identical experiments.

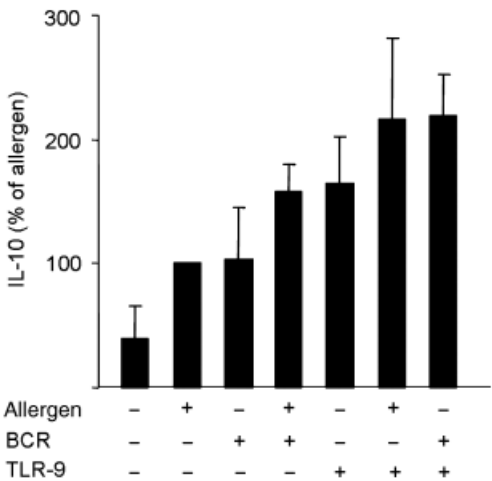


Fig. 4. Allergen extract-induced IL-10 expression in B cells signals through B cell receptor (BCR) and Toll-like receptor (TLR-9). Peripheral B cells were cultured for 72 h as indicated and IL-10 expression was quantified from the supernatants using ELISA. Data show arithmetic means±SEM from three individual experiments.

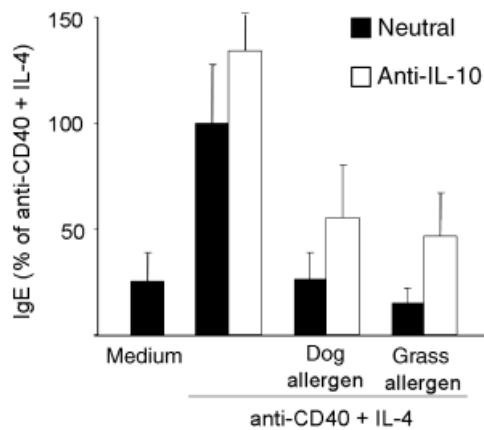


Fig. 5. Allergen extract-induced IL-10 inhibits IgE production of human B cells. Peripheral B cells were cultured with anti-CD40 and IL-4 for 10 days in the absence and presence of dog hair and grass pollen allergen (black bars; both at 10 000 SQ) with and without IL-10 neutralizing monoclonal antibody (white bars; 5 μ g/mL). Data show IgE in percentage of anti-CD40 and IL-4-stimulated control and are expressed as arithmetic means $\pm$ SEM from three individual experiments.

Allergen extract-induced interleukin-10 blocks anti-CD40 and interleukin-4 driven immunoglobulin E expression by B cells

IL-10 inhibits anti-CD40 and IL-4 driven IgE production of human B cells *in vitro* [17, 18]. The functional significance of allergen extract-induced IL-10 from B cells was investigated by adding dog hair or grass pollen allergen to highly purified CD19⁺ B cells stimulated by anti-CD40 and IL-4 for 10 days and detection of IgE in the supernatants (Fig. 5). IgE produced after anti-CD40 and IL-4 stimulation is set as 100%, corresponds to 3.1 ± 0.45 ng/mL of IgE. IgE production was strongly reduced in the presence of dog allergen (–65% of stimulated control) or grass pollen (–83%). To examine the role of allergen extract-induced IL-10, blocking antibodies against IL-10 were added to the cell culture at the beginning restored the IgE production in the presence of dog allergen by 53% or with grass pollen by 57% from the stimulated control. Addition of equal amount of an appropriate isotype control antibody did not alter the IgE production (data not shown). Allergen extract stimulation alone did not induce IgE (<0.5 ng/mL, data not shown). The other immunoglobulin subclasses IgG and IgA, determined from the same supernatants were not significantly altered by the allergen extracts with or without blocking of IL-10 (data not shown). These data show that allergen extract-induced IL-10 production of B cells inhibits the IgE production of human B cells.

Discussion

Here we show that common allergens, such as dog hair allergen, grass pollen or HDM induce IL-10 expression in

human memory B cells. The allergen extract-induced IL-10 producing B cells do not co-express the IL-4 inducible CD23 on the surface, whereas other activation markers such as CD69 are present. Analysis of underlying mechanisms indicated that activation of the BCR and/or TLR-9 are not exclusively involved. Functional relevance of B cell-derived IL-10 was shown here to be inhibition of anti-CD40 plus IL-4-mediated IgE expression in human B cells.

Our data show that only memory B cells (IgM[–]CD27⁺) expressed IL-10 upon allergen extract stimulation. The frequency of B cells expressing IL-10 was approximately 10% of all memory B cells, indicating that antigen-specific mechanisms are unlikely and that an intrinsic receptor in memory B cells is targeted. This is supported by the observation that in the supernatants of B cells activated in a polyclonal manner by antigen-receptor cross linking F(ab')₂ fragments, additional allergen extract stimulation increases the IL-10 expression.

A potential receptor responsible for allergen extract-mediated induction of IL-10 in human B cells, is TLR-9, which is prominent in memory B cells, but only slightly expressed in naïve B cells [22, 24]. TLR-9 is triggered by unmethylated DNA, which exists in prokaryotic, but also transcriptionally active eukaryotic cells. Our results show that TLR-9 triggering induces IL-10 in memory B cells (IgM[–]CD27⁺) more efficiently than in naïve B cells (IgM⁺CD27[–]). However, we demonstrated that allergen extracts increased IL-10 production in B cells with CpG2006 but also BCR activation. The BCR activation alone and the allergen extract stimulation results in a comparable IL-10 secretion. Furthermore, we performed a detailed analysis to determine whether the allergen extract-induced IL-10 secreting B cells might be specific for allergens. Staining of allergen-specific B cells was performed using fluorochrome-labelled allergens. The frequency of allergen-specific B cells was below the detection limit in allergic and non-allergic donors. Taken these observations into consideration together with the high frequency of allergen extract-induced IL-10 secreting B cells (7.2% of the memory B cells), we suppose that allergen extract-induced IL-10 secretion is rather not mediated through the antigen-receptor. Considering these observations we cannot exclude the possibility that allergen-dependent IL-10 production involves several signalling pathways.

The allergen extracts used in this study specifically induced IL-10 production in human B cells, in contrast to comparable amounts of LPS. This becomes evident by our flow cytometric analysis and also the data from supernatants of activated B cells. The latter finding also implies that the few contaminating cells, e.g. monocytes which express high levels of TLR-4 in contrast to B cells [23], were not involved in the observed allergen extract-mediated IL-10 expression.

CD23 modulates different steps leading to IgE synthesis. CD23 is induced by IL-4 [25] and IL-13 [26] and expressed on peripheral B cells of atopic individuals *ex vivo* [27]. Internalization and processing of allergen-IgE complexes via the CD23 enables B cells to present allergens via MHC-II to naïve T cells resulting in Th2 priming [3]. Also CD23-mediated antigen presentation of B cells can activate naïve T cells resulting in strongly increased specific antibody production [28–30]. In contrast, CD23 surface expression on B cells inhibits IgE production via a negative feedback loop [31–33]. Our data show that allergen extract-induced IL-10 secreting B cells do not co-express CD23, indicating that these cells are not activated by IL-4 or IL-13. These B cells may reduce Th2 cell differentiation by competing with CD23 expressing B cells and providing IL-10.

To gain insight into potential functions of allergen extract-induced IL-10 from human B cells, we investigated its impact on IgE production. Our experiments show that dog hair and grass pollen allergens strongly inhibit anti-CD40 and IL-4 driven IgE production of purified B cells. This effect was IL-10 dependent, as demonstrated by IgE up-regulation through addition of IL-10 neutralizing antibodies. These data suggest that allergens induce IL-10 in a distinct subset of memory B cells, which is involved in limiting Th2-mediated IgE production. However, allergens have different biological properties and it is also possible that other than IL-10-mediated mechanisms may play a role in IgE regulation. For example, Der p 1 cleaves surface CD23 to soluble CD23 [34]. The binding of IgE stabilizes the soluble CD23 trimers and thereby negatively influences IgE production. However, negative signalling pathway of CD23 remains unknown [1].

Furthermore, immunization with allergens in mice with plasmids containing cytosine–guanine dinucleotide motifs effectively inhibited IgE formation and inflammation [35]. Phosphorothioate- as well as phosphodiester-based CpG-ODNs have been described to decrease the synthesis of IgE from allergic donors previously [36–38]. Because IL-10 has an early down-regulating effect on IgE production through suppression of epsilon transcript expression [17] it is possible that the CpG-ODN-induced IL-10 from B cells is responsible for these reported observations.

The functional role of B cell-derived IL-10 in the modulation of type-I allergies is still not well known.

Evidence for T cell-derived IL-10 modulation of allergic immune responses was provided by analyses of PBMCs from repeatedly stung beekeepers or patients undergoing allergen-specific immunotherapy. Time frame of IL-10 expression by CD4⁺ helper cells overlapped with the induction of clinical tolerance [39]. IL-10 appear to limit different critical steps leading to the clinical manifestation of type-I allergies, namely antigen presentation and activation of T cells by dendritic cells, as well as IgE-

dependent activation of mast cells, survival and also cytokine production of eosinophils [8]. Recently, the mucosal and systemic IL-10 production during grass pollen-specific immunotherapy has been linked to the increase in allergen-specific IgG4 concentration [7, 40]. Although there is no information about the role of B cell-derived IL-10 on these type-I-specific processes *in vivo*, data indicate that IL-10 from any cell type can limit type-I allergic immune responses stated above, as shown in murine asthma models by applying transfected IL-10 expressing CD4T cells [41] or recombinant IL-10 directly to the lung of sensitized mice [8, 42]. Accordingly, it is possible that IL-10 producing B cells can control Th2 responses *in vivo*. Their anti-inflammatory potential was recently reported in a patient with ulcerative colitis, who suffered from a severe exacerbation after the depletion of peripheral B cells by anti-CD20 antibodies (rituximab), and local depletion of B cells and IL-10 [43]. Moreover, the primary onset of psoriasis, an IL-10 sensitive inflammatory skin disease, was reported after CD20⁺ B cell depletion in patients with diagnosed rheumatoid arthritis or systemic lupus erythematosus [44]; potentially by depletion of IL-10 producing B cells.

The key events leading to specific IgE expression in atopic individuals have not been defined yet. However, the analyses of phenotype and function of allergen extract-induced IL-10 producing B cells suggest a modulatory function on the allergen-specific immune response, namely by blocking IgE production and secretion of IL-10.

Acknowledgements

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The authors declare no conflict of interest.

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