

Original article

Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors

Background: Allergic diseases are characterized by the activation of the immune system and formation of immunoglobulin (Ig)E antibodies against normally innocuous environmental antigens, whereas IgG4 and IgA represent noninflammatory and blocking antibody isotypes. The T helper 2 (Th2) cells induce and T regulatory (Treg) cells suppress several features of allergic inflammation. Our aim was to investigate the role of allergen-specific T regulatory type 1 (Tr1) cells and CD4⁺CD25⁺ Treg cells and toll-like receptors (TLRs) on IgE, IgG4 and IgA production.

Methods: Germline or productive Ig-transcripts are determined by real-time reverse transcriptase-polymerase chain reaction, secreted Igs are measured by enzyme-linked immunosorbent assay and the frequency of Ig-producing plasma cells is investigated by enzyme-linked immunosorbent spot. Circulating CD4⁺CD25⁺ Treg cells and allergen-specific Tr1 cells are used.

Results: Both allergen-specific, interleukin-10-secreting Tr1 cells and CD4⁺CD25⁺ Treg cells from healthy individuals induced IgG4 and suppressed IgE production in peripheral blood mononuclear cells and purified B-cell cultures. In contrast, induction of IgA production is independent of T-cell help and the role of Tr1 or Treg cells is very limited, whereas it was highly induced by direct B-cell activation via TLR7 and 9.

Conclusions: These data suggest that T regulatory cells may contribute to the suppression of allergic diseases by suppression of IgE and induction of IgG4, whereas IgA production is enhanced by B-cell activation via TLR7 and TLR9.

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The physiopathology of allergic immune responses is complex and has been shown to be regulated by multiple factors. Hallmark of the allergic immune response is the development of allergen-specific T helper 2 (Th2) cells and their cytokines interleukin-4 (IL-4) and IL-13, which are responsible for immunoglobulin E (IgE) and IgG4 production by B cells (1). The type and amount of antibodies produced vary according to the type of allergen/antigen, involvement of T cells, prior history of antigen exposure, and anatomic site. Class switch recombination in B cells requires two signals. The first is delivered by cytokines, which target specific C_H genes for transcription, the second is delivered in the case of T-dependent antigens by interaction of CD40 on B cells with its ligand CD40L on activated T cells (2).

Patients suffering from asthma, allergic rhinitis and atopic dermatitis, have elevated serum levels of both total IgE and of IgE that is specific for the allergens that drive these diseases (3, 4). The induction of a tolerant state in peripheral T cells represents an essential step in healthy immune response to allergens in sensitized individuals (5). Peripheral T-cell tolerance observed in healthy individuals and during allergen-specific immu-

notherapy is characterized mainly by generation of allergen-specific T regulatory type 1 (Tr1) cells and increase in allergen-specific suppressive capacity of whole CD4⁺CD25⁺ T cells (6–8). This is accompanied by a significant increase in allergen-specific IgG4 and in some cases IgA and a decrease in IgE in the late stage of the treatment (4, 7, 9). Moreover, it has been shown that IL-10 decreased IL-4-induced IgE switching but augments IL-4-induced γ 4 transcript expression and IgG4 production (10).

Along with CD4 and CD25 expression, Treg cells are also associated with the transcription factor Foxp3 (11). Various autoimmune diseases such as arthritis, diabetes and X-linked immune dysregulation, polyendocrinopathy and enteropathy syndrome (IPEX) with hyper-IgE may develop spontaneously when CD4⁺CD25⁺ Treg cells are eliminated, suggesting a role for the control of IgE production (12). Immunoglobulin isotype switch, which was originally thought to be restricted to germinal centers of secondary lymphoid organs, can occur outside organized lymphoid tissues. IgE switch has been shown to take place in the nasal and bronchial mucosa of allergic rhinitis and asthma patients (13–15).

Human memory B cells express toll-like receptor 2 (TLR2), TLR6, TLR7, TLR9 and TLR10, but not TLR3 and TLR4 (16, 17). TLR7 and TLR9 recognize nucleic acids of microbial as well as of endogenous origin (18) and they are both present in endosomal compartments. As B cells are poorly phagocytic, but can efficiently internalize molecules that bind to the B-cell receptor (BCR), they can target RNA- or DNA-containing molecular complexes, which are capable of binding to the BCR, to TLR7 or TLR9 in the endosomal compartment. Consequently, TLR agonists can directly activate human memory, but not naive B cells and it has been suggested that this selective responsiveness may be a mechanism to maintain serological memory (18).

In the present study, we demonstrate that T regulatory cells regulate antibody isotypes, which may contribute to the suppression of inflammatory diseases by the induction of IgG4 and by the suppression of IgE. However, production of IgA is not influenced by regulatory T cells, but is strongly dependent on the innate immune system, particularly TLR7 and TLR9 activation of B cells.

Methods

Medium, antigen, cytokines and TLR-agonists

The medium used for 5-day cultures was RPMI 1640 supplemented as previously described (5). The medium used for 12-day cultures was additionally supplemented by 40 µg/ml of transferrin and 4 µg/ml of insulin (both from Sigma, Buchs, Switzerland). Recombinant *Dermaphagoides pteronyssinus* group 1 (Der p 1) of house dust mite (Allergopharma, Reinbek, Germany) and phospholipase A2 of bee venom (PLA, Sigma Chem. Co., Buchs, Switzerland) were used as allergens. They did not contain detectable amounts of LPS and were 99% pure. Recombinant human IL-4 was from Novartis, Basel, Switzerland, IL-10 from PeproTech, London, UK and anti-human IL-10 receptor from ATCC, Middlesex, UK. Soluble CD40L ligand (sCD40L) was produced from the transfected cell line 8-40-1, originally generated by Dr P. Lane (Birmingham, UK) and cultured for 3 days in CG medium (Vitromex GmbH, Vilshofen, Germany) and standardized according to highest IgE inducing capacity after 12 days culture of peripheral blood mononuclear cells (PBMC) co-stimulated with 50 ng/ml of IL-4 (19). Supernatants obtained from the corresponding untransfected cell line J558L (kindly provided by Dr M. Reth, University of Freiburg, Freiburg, Germany) were used as control.

Following TLR agonists were used: Pam3-Cys-Ser-(Lys)₄ for TLR2 (Calbiochem AG, Laufelfingen, Switzerland), poly(I:C) for TLR3 (Amersham Biosciences, Lausanne, Switzerland), *Escherichia Coli* derived lipopolysaccharide for TLR4 (Sigma), flagellin for TLR5 (Calbiochem AG), 3M-001 for TLR7 (3M Pharmaceuticals, Minneapolis, MN, USA), 3M-002 for TLR8 (3M Pharmaceuticals), CpG2006 for TLR9 (Microsynth GmbH, Balgach, Switzerland).

Purification of B cells, Tr1 and CD4⁺CD25⁺ Treg cells

Peripheral blood mononuclear cells were isolated by Ficoll (Seromed, Vienna, Austria) density gradient centrifugation from peripheral venous blood of healthy, nonallergic donors (based on history). B cells were isolated by depleting non-B cells by using a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD36, CD43 and

CD235a and anti-biotin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of B cells was more than 95%.

For the isolation of IL-10-secreting cells, PBMC were stimulated with 0.3 µM antigen in 5 ml of medium in six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) (5). After 12 h of stimulation in humidified 5% CO₂, cells were harvested and labeled with anti-IL-10/CD45 Ab-Ab conjugates (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were diluted with 37°C warm medium to a final concentration of 10⁶ cells/ml and allowed to secrete and capture IL-10. After capturing the secreted cytokine on their surface, cells were stained with PE-conjugated anti-IL-10 mAb. The cells were washed and resuspended in phosphate buffer saline, containing 0.5% bovine serum albumin and 2 mM EDTA, and magnetically labeled with anti-PE microbeads. After another washing step, labeled cells were purified (AutoMacs, Miltenyi Biotec, Bergisch Gladbach, Germany).

Purification of CD4⁺CD25⁺ Treg cells was performed in a two-step procedure. First, non-CD4⁺ cells were depleted with a cocktail of biotin-conjugated antibodies against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and glycophorin A and anti-biotin microbeads. In the second step, CD4⁺CD25⁺ T cells were directly labeled with anti-CD25 microbeads and isolated by positive selection from the pre-enriched CD4⁺ T-cell fraction. The cells were counterstained by FITC-labeled anti-CD4 and PE-labeled anti-CD25 mAb (Beckman Coulter, Fullerton, CA, USA) and analyzed in a flow cytometer (Epics XL; Beckman Coulter). The purity of CD4⁺CD25⁺ Treg cells was between 92.5% and 96.9%. High Foxp3 expression in CD4⁺CD25⁺ cells, purified by this method, has been previously reported (20).

Cell cultures

Peripheral blood mononuclear cells or pure B cells from nonallergic and healthy donors were incubated at 37°C either for 5 days [(reverse transcriptase-polymerase chain reaction (RT-PCR)) in cRPMI or for 12 days [(enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunosorbent spot (ELISPOT))] in insulin and transferrin supplemented cRPMI with IL-10-secreting Tr1 or CD4⁺CD25⁺ cells. After optimization of cell number, 2.5 × 10⁶ PBMC were cultured with 4000 isolated IL-10-secreting, Der p 1-specific T cells or with 33 000 CD4⁺CD25⁺ T cells in six-well plates. A total of 4000 Der p 1-specific, IL-10-secreting cells or 11 000 CD4⁺CD25⁺ T cells were co-cultured with 200 000 purified B cells in 96-well plates.

Determination of IgE, IgG4 and IgA by real-time PCR, ELISA and ELISPOT (refer to methods in the Supporting information)

Statistical analysis

Statistical analysis was performed using the paired Student's *t*-test and the Wilcoxon matched pairs signed ranks test. Changes less than two PCR cycles in real-time PCR were not taken into account for the analysis of significance.

Results

IL-10-secreting Tr1 cells suppress IgE and induce IgG4 production

Antibody synthesis and secretion in response to protein antigens are stimulated by CD40-mediated signals and

B-cell-activating cytokines expressed by T cells. Therefore, it was hypothesized that allergen-specific, IL-10-secreting Tr1 cells, which represent the dominant subset specific for common environmental allergens in healthy individuals, may also have an effect on Ig regulation. In the following experiments, we used IL-4 and sCD40L to induce IgE and IgG4, because IL-10-secreting Tr1 cells and IL-10 alone induced only very little IgG4 and no IgE (Fig. S1 in the Supporting information). Der p 1-specific, IL-10-secreting, Tr1 cells are isolated from PBMC of healthy individuals and their role on IgE and IgG4 production is investigated. As shown in Fig. 1A, Tr1 cells suppressed germline ϵ transcript expression and simultaneously, increased productive $\gamma 4$ mRNA expression in IL-4- and sCD40L-stimulated PBMC. Consistently, quantification of the secreted immunoglobulins also verified our findings. T regulatory type 1 cells decreased IgE- and enhanced IgG4-secretion (Fig. 1B). Similar

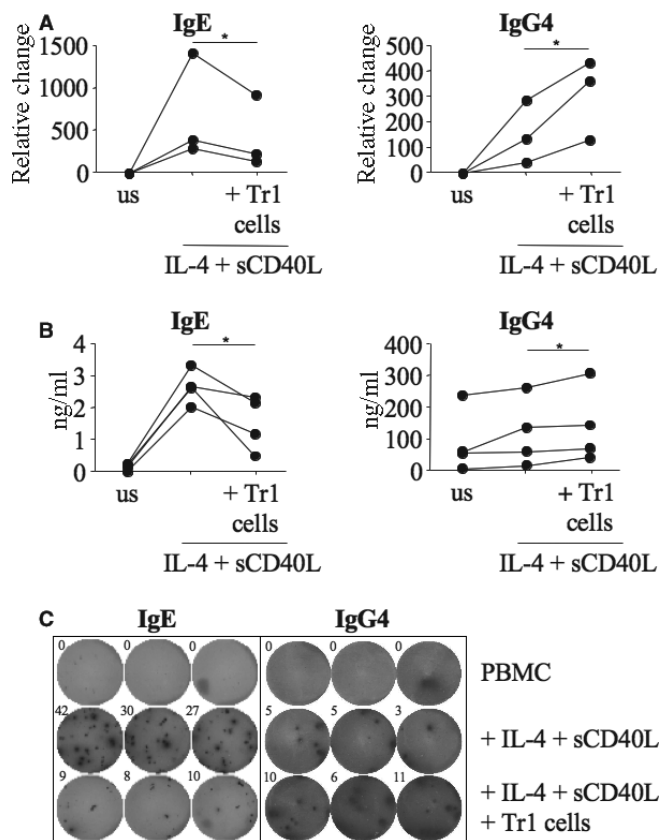


Figure 1. Allergen-specific, IL-10-secreting Tr1 cells suppress IgE, but induce IgG4. Der p 1-specific, IL-10-secreting Tr1 cells are isolated from PBMC from healthy donors and incubated together with PBMC. (A) Quantitative RT-PCR determined at day 5 ($n = 3$). (B) Supernatants analyzed for IgE and IgG4 at day 12 ($n = 4$). Conditions in the same experiment are linked with a line. (C) The frequency of IgE- and IgG4-secreting plasma cells measured at day 12 (one representative of three independent experiments is shown). * $P < 0.05$.

results are found considering the frequency of IgE- and IgG4-secreting plasma cells. T regulatory type 1 cells decreased the number of IgE-secreting plasma cells, but enhanced the number of IgG4-secreting plasma cells (Fig. 1C). For comparison, Der p 1 or bee venom phospholipase A2-specific IL-4-secreting and IFN- γ -secreting T cells were used in the same type of experiments. IL-4-secreting T cells significantly increased IgG4 and IgE production, whereas IFN- γ -secreting T cells did not affect IgE and IgG4 production (Fig. S2 in the Supporting information).

Differential effect of CD4⁺CD25⁺ Treg cells on IgE and IgG4 production by PBMC from healthy individuals

We next investigated the influence of CD4⁺CD25⁺ Treg cells on immunoglobulin production. Similar to Tr1 cells, human peripheral blood CD4⁺CD25⁺ Treg cells also secrete IL-10 (7). Incubation of IL-4- and sCD40L-stimulated PBMC with CD4⁺CD25⁺ Treg cells from healthy individuals inhibited germline ϵ transcript expression, but they tend to induce productive $\gamma 4$ transcript expression compared to CD4⁺CD25⁻ T cells (Fig. 2A). The same effects of CD4⁺CD25⁺ Treg cells were observed on the protein level. As shown in Fig. 2B, CD4⁺CD25⁺ Treg cells suppressed IgE secretion, whereas IgG4 production was enhanced by IL-4- and sCD40L-stimulated PBMC. Freshly purified CD4⁺CD25⁺ T cells did not induce IgE or IgG4 production in conditions where IL-4 and sCD40L were not used, suggesting that Th2 help is required for the production of these Ig isotypes (Fig. S1 in the Supporting information). Not surprisingly, also the frequency of IgE- and IgG4-secreting plasma cells was decreased or enhanced, respectively, when CD4⁺CD25⁺ Treg cells were added to the IL-4- and sCD40L-stimulated PBMC cultures (Fig. 2C). CD4⁺CD25⁻ T cells did not influence IL-4 and sCD40L-stimulated IgE or IgG4 production.

IL-10 counter regulates IgE and IgG4

On the basis of the results above and since IL-10 is one of the major cytokines of both Tr1 cells and CD4⁺CD25⁺ Treg cells, we next wanted to investigate the role of this cytokine on the Ig regulation. IL-10 decreased IL-4 and sCD40L-induced germline ϵ transcript expression (Fig. 3A). In contrast, IL-4 and sCD40L-induced productive $\gamma 4$ transcript expression is increased by IL-10. These effects of IL-10 could be observed in a dose-dependent manner in the range of 2–50 ng/ml. The findings in quantitative PCR are confirmed by determination of Igs in the supernatants of 12-day-cultures. Peripheral blood mononuclear cells stimulated with IL-4 and sCD40L showed decreased IgE secretion in the presence of IL-10 (Fig. 3B). In the same cultures, IL-10 synergized IL-4 and sCD40L-induced IgG4 secretion, whereas alone, it did induce only very small amounts of

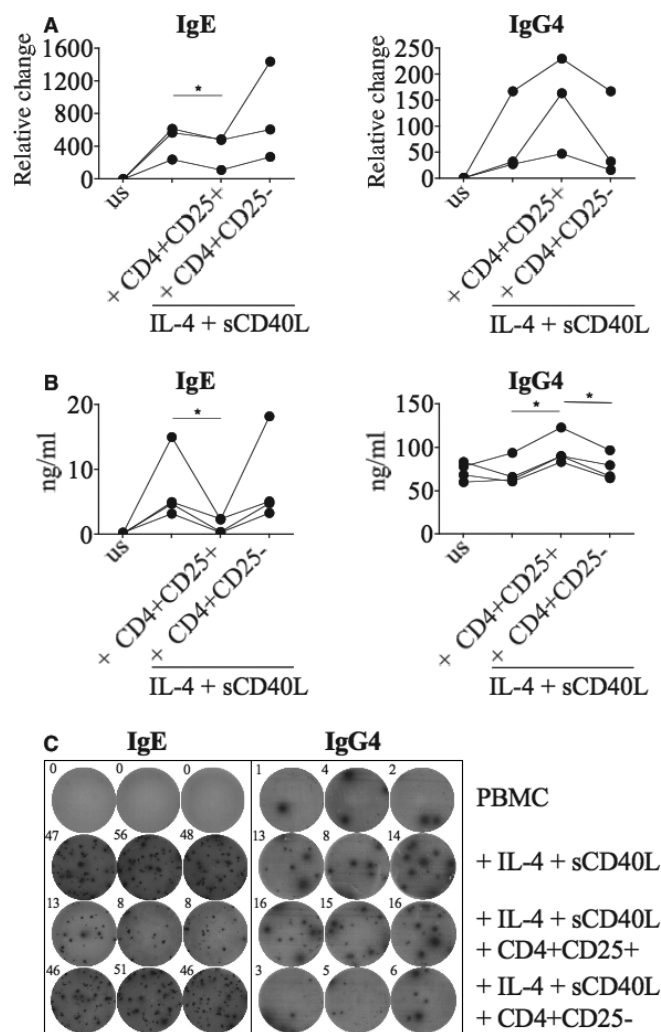


Figure 2. Suppression of IgE and induction of IgG4 by CD4⁺CD25⁺ Treg cells. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells are isolated from PBMC from healthy donors and cultured together with PBMC. (A) Quantitative mRNA for RT-PCR measured at day 5 ($n = 3$). (B) IgE and IgG4 concentrations in the supernatants at day 12 ($n = 4$). (C) The number of IgE- and IgG4-secreting B cells analysed at day 12 (one representative of three independent experiments is shown). * $P < 0.05$.

IgG4 (Fig. S1 in the Supporting information). Similarly, IL-10 reduced the frequency of IgE-secreting plasma cells, whereas the IgG4-secreting plasma cell-frequency is enhanced (Fig. 3C). In unstimulated conditions, the frequency of IgE-producing plasma cells was practically negative. Only one spot per 50 000 cells was observed in nine different experiments. The frequency of IgG4-producing plasma cells ranged between 0 and 4 spots per 50 000 resting PBMC. In IL-4- and sCD40L-stimulated cultures IgE spots increased to 27–59, IgG4 spots increased to 1–14 spots. Interestingly, the secreted protein of IgG4 was relatively high compared to IgE, suggesting utilization of IgE in cultures, such as binding to CD23

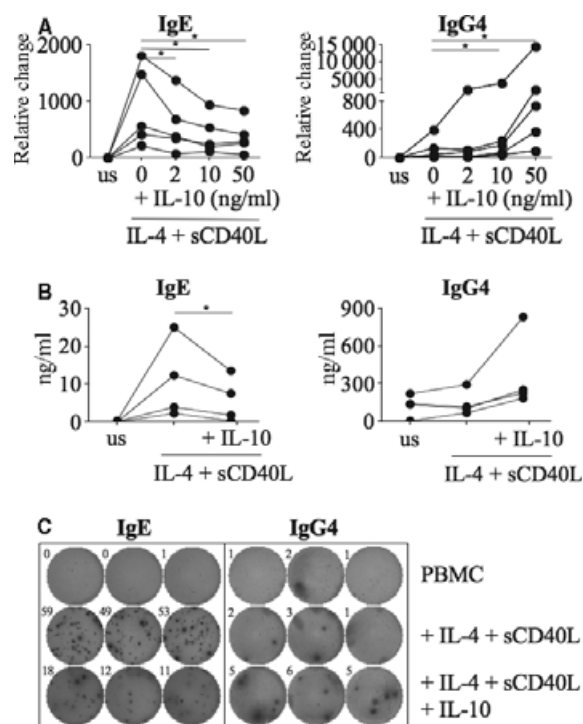


Figure 3. Interleukin-10 suppresses IgE and induces IgG4 production in PBMC. Human PBMC from healthy donors are cultured with different concentrations of IL-10. (A) Quantitative RT-PCR measured after 5 days of incubation ($n = 5$). (B) Immunoglobulin concentrations in the supernatants determined at day 12 ($n = 4$). (C) The frequency of IgE- and IgG4-secreting plasma cells measured at day 12 (one representative of three independent experiments is shown). (B, C) IL-10 was used at 50 ng/ml. * $P < 0.05$.

(21). Blocking of the IL-10 receptor in CD4⁺CD25⁺ Treg co-cultures demonstrated that their suppression effect on IgE and their induction effect on IgG4 are diminished (Fig. S3A in the Supporting information). To strengthen the role of IL-10 as a soluble factor in the induction of IgG4, we performed transwell experiments, in which IL-10-secreting Tr1 cells were cultured in the upper well of the transwells and PBMC were cultured in the bottom of the transwells. IL-10-producing Tr1 cells induced IgG4 production without any requirement of cell-to-cell contact (Fig. S3B in the Supporting information). In addition, IL-10 or IL-10R α -chain were blocked by mAbs in the presence of IL-10. Immunoglobulin G4, which was significantly induced by IL-10 was almost 100% inhibited either by blocking the cytokine itself or its receptor (Fig. S3C in the Supporting information).

Direct effect of Tr1 cells, CD4⁺CD25⁺ Treg cells and IL-10 on IgE and IgG4 production in pure B cells

To eliminate indirect effects of other cells in PBMC, we investigated the impact of IL-10-secreting Tr1 cells,

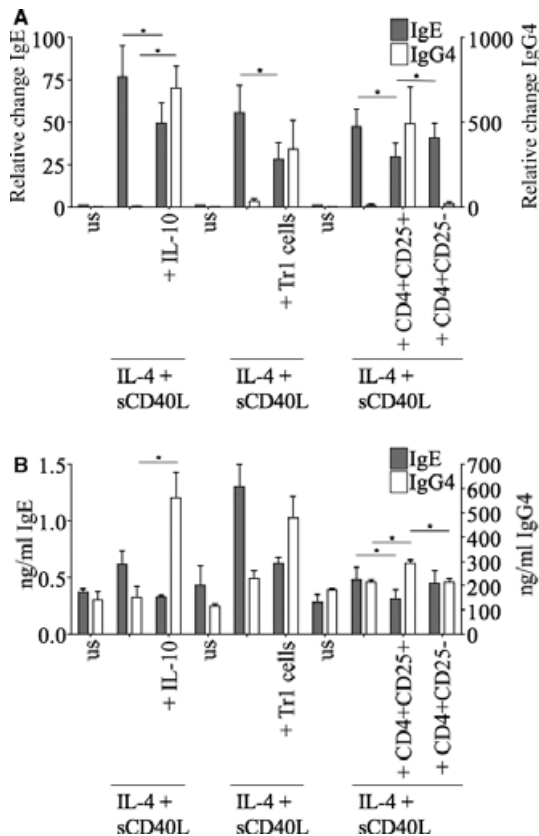


Figure 4. Interleukin-10 (IL-10)-secreting Tr1 cells and CD4⁺CD25⁺ Treg cells regulate B cells. B cells, IL-10-secreting Tr1 cells, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells are isolated from PBMC from healthy individuals. (A) Quantitative RT-PCR determined after 5 days of incubation of B cells with IL-10 (50 ng/ml), Tr1 cells, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells (mean values $\pm$ SE; $n = 9$). (B) Immunoglobulin concentrations measured at day 12 in the supernatants (mean values $\pm$ SE; $n = 9$). * $P < 0.05$.

CD4⁺CD25⁺ Treg cells and IL-10 on purified B cells. B cells were cultured in the presence of IL-4 and sCD40L. As observed in PBMC, addition of Der p 1-specific Tr1 cells, CD4⁺CD25⁺ Treg cells or IL-10 decreased germ-line ϵ transcript expression. Productive $\gamma 4$ transcript expression was increased by Tr1 cells, CD4⁺CD25⁺ Treg cells and IL-10 (Fig. 4A). Considering the Ig-protein level, IgE secretion was diminished in the presence of Tr1 cells, CD4⁺CD25⁺ Treg cells or IL-10, but IgG4 secretion was strongly enhanced (Fig. 4B).

Therefore, we conclude, that antigen-specific, IL-10-secreting Tr1 cells and CD4⁺CD25⁺ Treg cells are able to reduce IgE production at the mRNA-, the protein- and the plasma cell frequency-level. Simultaneously, antigen-specific, IL-10-secreting Tr1 cells and CD4⁺CD25⁺ Treg cells can efficiently induce IgG4 production. The data is confirmed in 50 different experiments demonstrated in Figs 1–4.

IgA is induced by TLR7 or 9 stimulation but not by T-cell help

Because neither Tr1 cells nor CD4⁺CD25⁺ Treg cells nor CD4⁺CD25⁻ T cells nor IL-10 showed any influence on IgA production in PBMC as well as in pure B-cell cultures (Fig. S4A in the Supporting information), we then investigated an array of TLR agonists for their capacity to induce IgA. Peripheral blood mononuclear cells were cultured in the presence of different TLR agonists. While stimulation of TLR2, 3, 4, 5, or 8 was not able to influence the IgA production (Fig. S4B in the Supporting information), triggering of TLR7 or 9 highly enhanced both the mRNA level and the secretion of IgA compared to IgE and IgG4 (Figs 5A and B). Interestingly, the addition of IL-4 and sCD40L to the cultures suppressed TLR7 or 9 induced IgA production, but upregulated IgE and IgG4 (Fig. S4B in the Supporting information). Although dose dependent optimization was performed in preliminary experiments, TLR9 stimulation by type B CpG has always been the most potent stimulus compared to TLR7 for the induction of IgA. As a control, we also used type A CpGs, which could not induce IgA (Fig. S5 in the Supporting information). We conclude that the IgA production is not influenced by Tr1 and CD4⁺CD25⁺ regulatory T cells, but rather induced by the innate immune system via activation of TLR7 or 9 stimulation.

Discussion

In the present study, we demonstrate that the function of T regulatory cells is not only to suppress Th1 or Th2 cell responses, but that those cells also have direct influence on B cells. Tr1 cells and CD4⁺CD25⁺ Treg cells suppressed IgE and induced IgG4. Both regulatory T-cell subsets also lowered IgE-secreting, and simultaneously augmented the IgG4-secreting plasma cell-frequency. In lymph nodes and tonsils, Foxp3⁺ Treg cells are present in B-cell areas where T–B-cell interaction and humoral immune responses occur, and can directly suppress B-cell Ig production and class switch recombination without a prerequisite to suppress Th cells. This is supported by studies, which show decreased Treg cell function or number in several immune mediated diseases in humans and mouse models with increased autoantibody production (22, 23).

Studies on the mechanisms of immune responses to allergens have demonstrated that Treg cells are dominant in healthy individuals (5, 24). If a detectable immune response is mounted, Tr1 cells specific for common environmental allergens, consistently represent the dominant subset. Healthy and allergic individuals exhibit the Th1, Th2 and Tr1 type, allergen-specific subsets in different proportions (5). Accordingly, a change in the dominant subset and the balance between Th2 and Treg cells may lead to either allergy development or recovery.

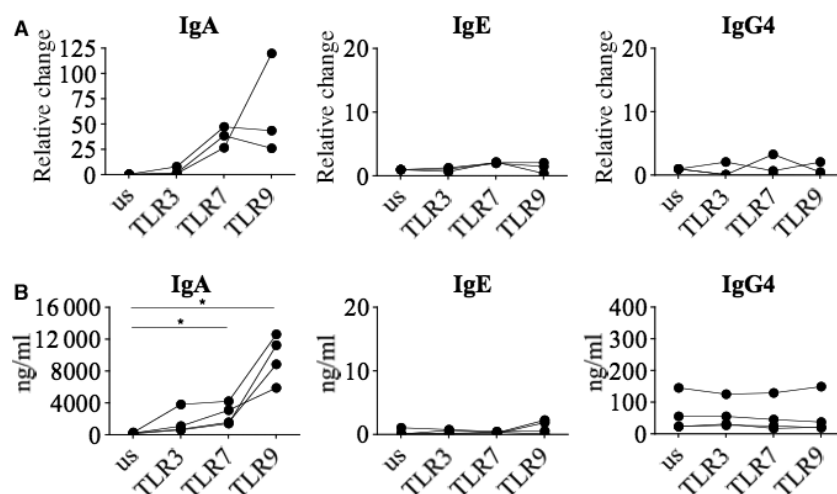


Figure 5. Induction of IgA requires TLR7 and 9 triggering. Peripheral blood mononuclear cells from healthy individuals are stimulated with TLR3, 7 or 9 ligands. (A) Quantitative RT-PCR determined after 5 days of incubation ($n = 3$). (B) IgA, IgE and IgG4 concentrations in the supernatants measured after 12 days ($n = 4$).

CD4⁺CD25⁺ Treg cells have been associated with the spontaneous remission of Cow's milk allergy. Children who outgrew their allergy (tolerant children) had higher frequencies of circulating CD4⁺CD25⁺ T cells and decreased *in vitro* proliferative responses to bovine beta-lactoglobulin in PBMC compared with children who maintained clinically active allergy (25). Several studies have suggested that children with a cat at home have a decreased risk of sensitization and asthma. Children exposed to more than 20 µg of Fel d 1 per 1 g of house dust display an IgG4 antibody response to Fel d 1 without IgE antibody. This modified Th2 response is not associated with symptoms and has been regarded as a form of immunological tolerance (26).

Although peripheral T-cell tolerance is rapidly induced during specific immunotherapy (SIT), there is no evidence for B-cell tolerance in the early course (27). Especially IgG4, which increases during the course of SIT or natural high dose exposure, is thought to capture the allergen before reaching the effector cell-bound IgE, and thus to prevent the activation of mast cells and basophils (28). Another study shows that costimulation of FcγRII and FcεRI inhibits human basophil FcεRI-mediated histamine release, IL-4 production and Ca²⁺ mobilization (29). Allergen-specific IgG4 may be directed against the same epitopes as IgE, resulting in direct competition for allergen binding and a 'blocking' effect (30). It has been proposed that successful specific immunotherapy is associated with an increase in IgG blocking activity that is not solely dependent on the quantity of IgG antibodies (30, 31).

The noninflammatory role for IgG4 may be because the IgG4 hinge region has unique structural features that result in a lower affinity for certain Fcγ receptors and the ability to separate and pair again, leading to bi-specific antibodies that are functionally monomeric (32, 33).

Furthermore, IgG4 does not fix complement and is unable to form large immune complexes. In an allergen-specific immunotherapy study, in which well-defined recombinant allergen mixtures were used, all treated persons developed very strong allergen-specific IgG4 and also increased IgG1 antibody responses. Some patients, who were not initially sensitized to *Phleum pratense* group 5 (Phl p 5), developed strong specific IgG4, but not IgE antibody responses to that allergen (34).

Interleukin-10 is a potent suppressor of both total and allergen-specific IgE, while it simultaneously increases IgG4 production (6, 10). Thus, IL-10 not only generates tolerance in T cells; it also regulates specific isotype formation and skews the specific response from an IgE to an IgG4 dominated phenotype. The healthy immune response to Der p 1 demonstrated increased specific IgA and IgG4, small amounts of IgG1 and almost undetectable IgE antibodies in serum (7). House dust mite-SIT did not significantly change specific IgE levels after 70 days of treatment; however, a significant increase in specific IgA, IgG1 and IgG4 was observed (7). Most probably the decrease in IgE/IgG4 ratio during allergen-SIT is a feature of skewing an allergen-specific Th2 to a Treg cell predominance. However, although Treg cell generation happens within days, a significant decrease in IgE/IgG4 ratio occurs after several months. The reason for the long-time gap between the change in T-cell subsets, but not IgE/IgG4 levels is not easily explainable by the half-life of IgE (2.5 days) and IgG4 (23 days) antibodies. In this context, bone marrow-residing plasma cells with very long lifespan could be responsible for this effect (35). To increase the efficacy of allergen-SIT a search for good adjuvants is efficiently being pursued. Usage of TLR7/9 triggering substances together with recombinant proteins may have a beneficial effect in allergen-specific immunotherapy. In this way, the blocking antibody isotype IgA is

expected to increase simultaneously and support or even synergize the advantageous effects of IgG4 (31, 36).

In this study, we demonstrate that IgA production is neither influenced by IL-10 nor by IL-10-secreting Tr1 cells nor by CD4⁺CD25⁺ Treg cells. This antibody isotype is particularly regulated by activation of the innate immune system. Addition of TLR7 or 9 agonists to PBMC cultures highly upregulated IgA production and secretion, whereas T cell-help to B cells seems not to be necessary, since Tr1 cells, CD4⁺CD25⁺ Treg cells as well as IL-4 and sCD40L did not have any effect on IgA. TGF- β , which is also produced by regulatory T cells, is well known as a specific switch factor for IgA, but only during the first 24 h. Once B cells have switched, IgA synthesis is inhibited by TGF- β (37), which is in agreement with our results as we measured productive transcripts of IgA. Recent evidence indicates that TLR stimulation on B cells can control immunoglobulin isotype switch. TLR and MyD88 are required for class switching to the pathogenetic IgG2a and IgG2b isotypes, but not for the development of IgM autoantibodies (38). Furthermore, CpG, but not LPS, upregulates T-bet expression in B cells and induces IgG2a, whereas it decreases IgG1 and IgE production (39, 40). In human B cells, TLR9 stimulation by CpG-DNA, in association with IL-10, initiates germline immunoglobulin heavy chain constant region C γ 1, C γ 2 and C γ 3 gene transcription (41). Besides having a direct effect on B cells, TLR agonists can affect B-cell differentiation and isotype switch indirectly through activation of DCs and subsequent release of IFN- α and IL-6. Recently, it has been shown that gut DCs induce IgA class switch by synergistic effects of retinoic acid, IL-6 and IL-5 (42), however, the role of TLRs in DC-mediated IgA induction remains to be elucidated. In another study, IFN- α -, IFN- γ -, LPS- or CD40L-stimulated human dendritic cells up-regulated B cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), which induced together with IL-4, IL-10 or TGF- β CD40-independent class-switch recombination to C γ , C α or C ϵ in B cells (43). In addition, TLR ligands and APRIL were shown to synergize to further enhance IgA2 class switching (44). Moreover, it has been demonstrated that inducible-nitric-oxide-synthase (iNOS) induces TGF- β RII on B cells and controls T-cell-dependent IgA class-switch recombination. Furthermore, MALT DCs express iNOS in response to the recognition of commensal bacteria by TLRs, which in turn leads to the production of BAFF and APRIL and subsequent T-cell-independent IgA class-switch recombination in B1 cells (45).

If the mechanism by which regulatory T cells act on B cells consists of physical cell-to-cell contact via intercellular adhesion molecule 1 (ICAM-1), TGF- β (46), cytotoxic T cell-associated antigen-4 (CTLA-4) (47), glucocorticoid-induced tumor necrosis factor receptor (48), programmed death-1 (PD-1) (49) and OX40 (CD134) (50), or, as very recently shown, via cyclic

adenosine monophosphate and gap junctions (51), or if it is more an paracrine mechanism with IL-10 and TGF- β as soluble mediators, is not known yet. It appears however, that this antibody isotype regulation is partially dependent on IL-10 as demonstrated in our inhibition experiments with anti-IL-10 and anti-IL-10 receptor-antibodies and the transwell experiment (Fig. S3 in the Supporting information).

We conclude that peripheral tolerance utilizes multiple mechanisms to suppress allergic inflammation. Apparently, Treg cells contribute to the control of allergen-specific immune responses in several ways: suppression of antigen-presenting cells that support the generation of effector Th2 and Th1 cells; suppression of Th2 and Th1 cells; suppression of mast cells, basophils and eosinophils; interaction with resident tissue cells and remodeling (52). In addition to the above mechanisms, the present study demonstrates suppression of IgE and induction of the noninflammatory antibody isotype IgG4 by Tr1 and CD4⁺CD25⁺ Treg cells.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Figure S1 IgG4 and IgE are not strongly induced by IL-10, IL-10-secreting Tr1 cells or CD4⁺CD25⁺ Treg cells without IL-4 and sCD40L. PBMC from healthy individuals were incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or CD4⁺CD25⁺ Treg cells. (A) Quantitative RT-PCR determined after 5 days of incubation (mean values $\pm$ SE; n = 4). (B) The IgE and IgG4 concentrations in the supernatants measured after 12 days of incubation (mean values $\pm$ SE; n = 4). **Figure S2** Regulation of IgE and IgG4 by IL-4- and IFN- γ -secreting cells. Der p 1 or bee venom phospholipase A2-specific IL-4-secreting and IFN- γ -secreting T cells were used as controls. IL-4-secreting T cells significantly increased IgG4 and IgE production, whereas IFN- γ -secreting T cells did not affect IgE and IgG4 production. **Figure S3** Role of IL-10 in suppression of IgE and induction of IgG4 by Treg cells. B cells from healthy individuals are incubated with CD4⁺CD25⁺ Treg cells or with CD4⁺CD25⁻ T cells. Quantitative RT-PCR determined after 5 days of incubation. (A) IgE germline transcripts and IgG4 productive transcripts (one repre-

sentative experiment). (B) IL-10-secreting Tr1 cells were cultured in the upper compartment of the transwells and PBMC were cultured in the lower compartment. IL-10-producing Tr1 cells induced IgG4 production without any requirement of cell-to-cell contact (data represent 2 independent experiments). (C) In PBMC, IL-10 or IL-10RA-chain was blocked by mAbs in the presence of IL-10. IgG4, which was significantly induced by IL-10 was strongly inhibited either by blocking the cytokine itself or its receptor (data represent 3 independent experiments) * $P < 0.05$. IC: isotype control.

Figure S4 IgA is elicited by stimulation of TLR7 or 9 but not by IL-10, IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells. (A) PBMC or pure B cells from healthy individuals are incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells. Quantitative RT-PCR determined after 5 days of incubation (mean

values $\pm$ SE; $n = 3-5$). (B) PBMC from healthy individuals are stimulated with TLR2, 3, 4, 5, 7, 8 or 9 ligands. Quantitative RTPCR determined after 5 days of incubation ($n = 3$). IL-4 and sCD40L, which upregulated IgE and IgG4 suppressed TLR7 and TLR9 induced IgA.

Figure S5 IgA was determined after stimulation of PBMC with type B CpG2006 and type A CpG10 and CpG2216 (data represent 3 independent experiments). CpG2006 was found to be the most potent inducer of IgA. In contrast, induction of IgA by A type CpGs was very weak. IgA mRNA was detected on day 5.

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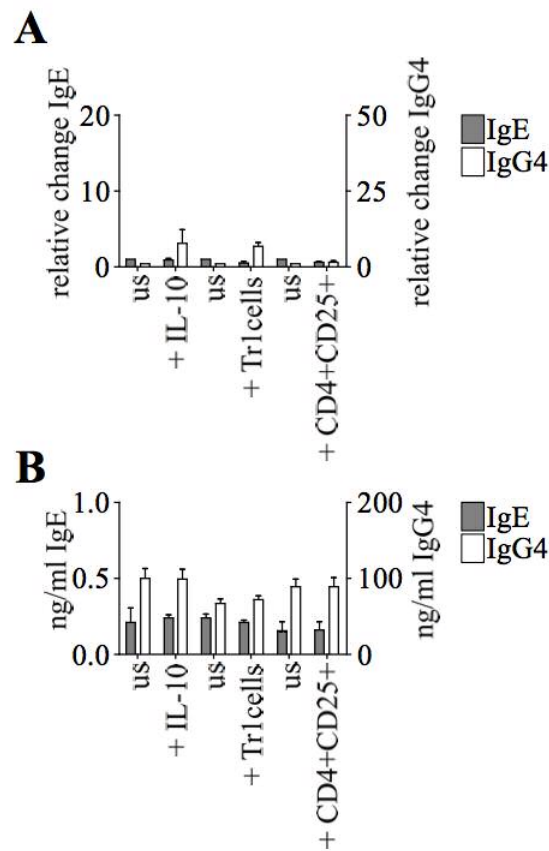


Fig. S1. Meiler et al.

IgG4 and IgE are not strongly induced by IL-10, IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells without IL-4 and sCD40L. PBMC from healthy individuals were incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells. (A) Quantitative RT-PCR determined after 5 days of incubation (mean values $\pm$ SE; n=4). (B) The IgE and IgG4 concentrations in the supernatants measured after 12 days of incubation (mean values $\pm$ SE; n=4).

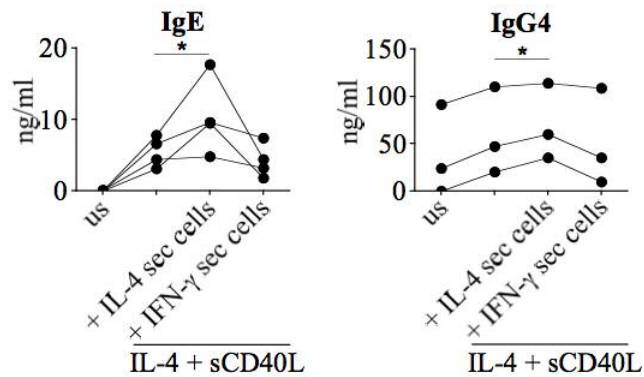


Fig. S2. Meiler et al.

Regulation of IgE and IgG4 by IL-4- and IFN- γ -secreting cells. Der p 1 or bee venom phospholipase A2-specific IL-4-secreting and IFN- γ -secreting T cells were used as controls. IL-4-secreting T cells significantly increased IgG4 and IgE production, whereas IFN- γ -secreting T cells did not affect IgE and IgG4 production.

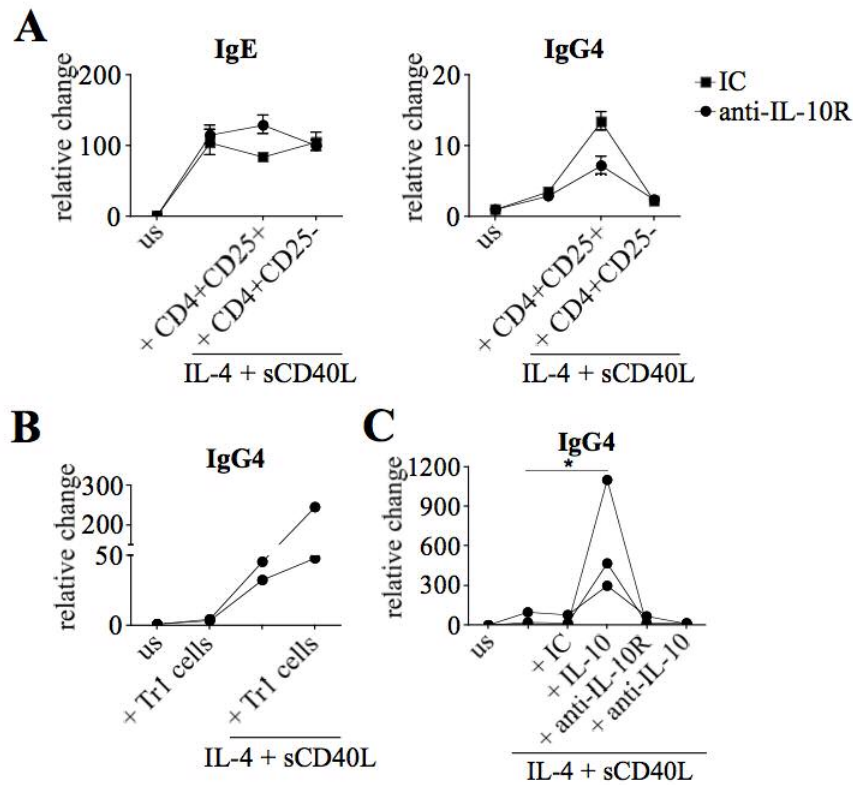


Fig. S3. Meiler et al.

Role of IL-10 in suppression of IgE and induction of IgG4 by Treg cells. B cells from healthy individuals are incubated with CD4+CD25+ Treg cells or with CD4+CD25- T cells. Quantitative RT-PCR determined after 5 days of incubation. (A) IgE germline transcripts and IgG4 productive transcripts (one representative experiment). (B) IL-10-secreting Tr1 cells were cultured in the upper compartment of the transwells and PBMC were cultured in the lower compartment. IL-10-producing Tr1 cells induced IgG4 production without any requirement of cell-to-cell contact (data represent 2 independent experiments). (C) In PBMC, IL-10 or IL-10R α -chain was blocked by mAbs in the presence of IL-10. IgG4, which was significantly induced by IL-10 was strongly inhibited either by blocking the cytokine itself or its receptor (data represent 3 independent experiments) * $p < 0.05$. IC: isotype control.

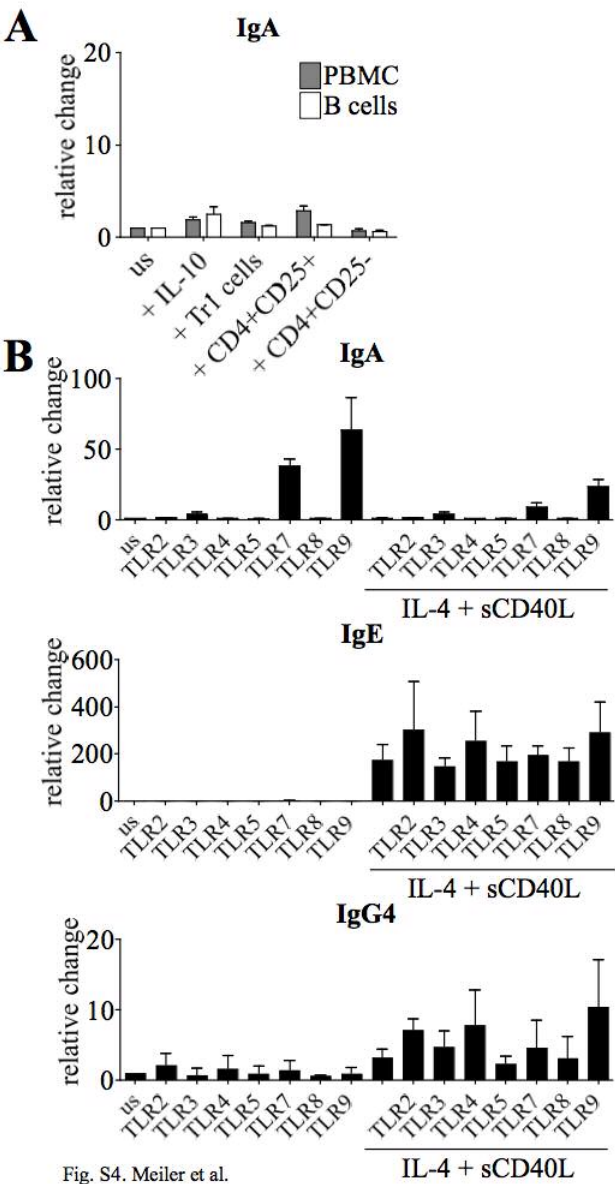


Fig. S4. Meiler et al.

IgA is elicited by stimulation of TLR7 or 9 but not by IL-10, IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells. (A) PBMC or pure B cells from healthy individuals are incubated with IL-10 (50 ng/ml), IL-10-secreting Tr1 cells or CD4+CD25+ Treg cells. Quantitative RT-PCR determined after 5 days of incubation (mean values $\pm$ SE; n=3-5). (B) PBMC from healthy individuals are stimulated with TLR2, 3, 4, 5, 7, 8 or 9 ligands. Quantitative RT-PCR determined after 5 days of incubation (n=3). IL-4 and sCD40L, which upregulated IgE and IgG4 suppressed TLR7 and TLR9 induced IgA.

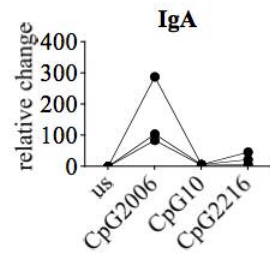


Fig. S5. Meiler et al.

IgA was determined after stimulation of PBMC with type B CpG2006 and type A CpG10 and CpG2216 (data represent 3 independent experiments). CpG2006 was found to be the most potent inducer of IgA. In contrast, induction of IgA by A type CpGs was very weak. IgA mRNA was detected on day 5.