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**Title:** Regulatory B cells in allergic asthma and schistosomiasis: controlling inflammation  
**Issue Date:** 2015-02-12
CD24^{hi}CD27^{+} B cells from patients with allergic asthma have impaired regulatory activity in response to lipopolysaccharide

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Clinical and Experimental Allergy, 2014 April; 44(4):517-28
Abstract

**Background** Regulatory B cells have been identified that strongly reduce allergic and auto-immune inflammation in experimental models by producing IL-10. Recently, several human regulatory B-cell subsets with an impaired function in auto-immunity have been described, but there is no information on regulatory B cells in allergic asthma.

**Objective** In this study, the frequency and function of IL-10-producing B-cell subsets in allergic asthma were investigated.

**Methods** Isolated peripheral blood B cells from 13 patients with allergic asthma and matched healthy controls were analyzed for the expression of different regulatory B-cell markers. Next, the B cells were activated by lipopolysaccharide (LPS), CpG or through the B-cell receptor, followed by co-culture with endogenous memory CD4⁺ T cells and house dust mite allergen Derp1.

**Results** Lower number of IL-10-producing B cells were found in patients in response to LPS, however, this was not the case when B cells were activated through the B-cell receptor or by CpG. Further dissection showed that only the CD24⁺CD27⁺ B-cell subset was reduced in number and IL-10 production to LPS. In response to Derp1, CD4⁺ T cells from patients co-cultured with LPS-primed total B cells produced less IL-10 compared to similar cultures from controls. These results are in line with the finding that sorted CD24⁺CD27⁺ B cells are responsible for the induction of IL-10⁺ CD4⁺ T cells.

**Conclusions** Taken together, these data indicate that CD24⁺CD27⁺ B cells from allergic asthmatic patients produce less IL-10 in response to LPS leading to a weaker IL-10 induction in T cells in response to Derp1, which may play a role in allergic asthma.
Weaker regulatory B-cell function in allergic asthma

Introduction

Allergic asthma (AA) is a reversible obstructive lung disease, in which cross-talk between inflammatory cells and structural lung cells is central. There is a negative association between reduced exposure to infectious agents and increased prevalence of asthma and allergy in affluent populations (1), leading to the hypothesis that childhood exposure to environmental microbes helps to prevent allergy (2). Abundant early-life microbial exposure, such as to farm animals, unpasteurized milk products and bacterial DNA and endotoxin (lipopolysaccharide, LPS), is thought to instruct a robust development of the regulatory arm of the immune system, preventing uncontrolled inflammatory responses to allergens (2-6).

In healthy individuals, immune responses to allergens are either absent or characterized by an enhanced production of IL-10. Whereas (regulatory) T cells are known for their capacity to produce IL-10 (7), IL-10 is also produced by other cell types, including B cells. Interestingly, initial studies using adoptive transfers in auto-immunity models have shown that IL-10-producing B cells have a strong potential to reduce inflammation and, therefore, are called regulatory B (Breg) cells (8-11). Importantly, recent mouse studies have demonstrated that Breg cells can reduce allergic inflammation in both anaphylactic and airway inflammation models by virtue of their IL-10 production and the induction of Treg cells (12-15).

Various Breg subsets in humans, characterized by the expression of CD24hiCD38hi or by CD24hiCD27+, have been described and show a reduced function in certain auto-immune diseases, such as systemic lupus erythematosus (SLE) (16;17). During chronic hepatitis B virus infection, CD24hiCD38hi B cells were described to produce IL-10 and thereby suppress antigen-specific CD8+ T cell responses (18). In addition, an enhanced CD1d+ Breg subset was described in both schistosome-infected Gabonese individuals (14) and in mixed helminth-infected Brazilian multiple sclerosis (MS) patients which could inhibit auto-reactive T cell responses in vitro (19). Additionally, a reduced IL-10 production of B cells from MS patients in response to CD40 ligation and B-cell receptor (BCR) cross-linking was reflected mostly in the CD27- naive B cells (20). Taken together, different human Breg subsets may exist and for some their function is impaired in certain auto-immune diseases.

Several articles have studied the signals that control regulatory B cell number and/or activity, and have identified both BCR triggering and TLR ligation as important pathways to induce IL-10 production and functional regulatory activity. In this respect, TLR2 and 4 have been shown to promote IL-10-producing B cells in different infection models: for example, extracts from Leishmania major or Brugia malayi microfilaria use TLR4 (21), Helicobacter felis use TLR2 (22) and in Salmonella typhimurium infections MyD88-dependent signaling in B cells is crucial for IL-10 induction (23).
So far, no information is available on the presence of regulatory B cells in individuals with allergic asthma (AA) or on TLR or BCR-related pathways that can promote their development. As abundant exposure to bacterial products in the farm studies (and in particular to endotoxin) is linked to protection against allergic inflammation and bacterial products promotes IL-10-producing B cells in animal models, we focused on LPS and CpG as TLR stimuli to investigate its IL-10-inducing capacity in human B cells. Therefore, we here describe the Breg cell frequency in peripheral blood B cells from patients with AA compared to healthy controls (HC) and investigated the capacity to produce IL-10 in response to LPS, CpG or to BCR triggering and their ability to influence T-cell cytokine production.
Materials and methods

Study population
Non-smoking (or ex-smoking for more than 1 year, less than 10 pack-years) AA patients with house dust mite (HDM) allergy and control (n=13; age: 19-45 years; Table 1) were subjected to asthma control questionnaires (ACQ; based on (24)), spirometry, airway hyperreactivity (AHR) tests in response to metacholine (PC_{20} metacholine); skin prick tests (SPT) for HDM, tree and grass pollen, cat, dog and Aspergillus. Nitric oxide (FeNO) levels were analyzed in exhaled breath, total serum IgE and allergen-specific IgE for HDM, tree and grass pollen, cat, dog, horse and Aspergillus (Phadiatop) were measured in serum. Inclusion criteria for healthy controls: PC_{20} >19 mg/ml, negative SPT for all antigens and total IgE <30 IU/ml. Inclusion criteria for patients with AA: PC_{20} <9.6 mg/ml and a positive SPT for at least HDM (wheal >5 mm), total IgE ≥30 IU/ml and detectable specific IgE for at least HDM (> 0.7 kU/l). Patients with AA (seven out of 13) using inhaled corticosteroids or combination therapy were asked to cease their medication 2 weeks before blood taking. Ten healthy control subjects donated fresh blood samples twice. In addition, peripheral blood from healthy laboratory donors were used to perform some validation experiments (secretion assays, signaling and ELISA). The study was approved by the Medical Ethical Committee LUMC (P09.170) and performed according to the declaration of Helsinki. Written informed consent was given by all participants.

B- and T-cell isolation and flowcytometry
PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation in 50 ml tubes (Greiner Bio-One) from heparinized blood. B cells with a purity of ~98% were isolated by anti-CD19 MicroBeads (Miltenyi Biotec). The remaining cells were collected for further isolation of memory CD45RO^{+}CD4^{+} T cells using MicroBeads (Miltenyi Biotec). PBMCs or isolated B cells were stained in 96-well V-bottom plates (Greiner Bio-One) for CD1d-PE (CD1d42, BD), CD5-PercPeFluor710 (UCHT2, eBioscience), CD20-APC-eFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, Biolegend), CD27-APC (L128, BD), CD38-FITC (HIT2, BD), CD19-Pacific Blue (HIB19, Biolegend) and TLR4-biotin (HTA125, Biolegend) in combination with streptavidin-Qdot525 (Invitrogen) and FcyR-binding inhibitor (eBioscience) to define the different Breg cell subsets. CD20, which was equally expressed as CD19 on all B cell (subsets), was used to define B cells because of the MACS CD19 isolation procedure. Fluorescence minus one (FMOs) were used for proper gate setting for all markers.

In a subset of healthy control donors, cells were stained for CD24-PeCy7 (ML5, Biolegend) and CD27-APC (L128, BD Biosciences) after CD19^{+} B cell isolation and sorted into CD24^{hi}CD27^{+} and remaining B cells using FACSariaIII flowcytometer (BD Biosciences).
B-cell stimulation and intracellular staining for IL-10

B cells (1 x 10^5) were stimulated with 100 ng/ml ultra-pure LPS (Invitrogen), 5 µg/ml CpG ODN2006 (Invitrogen) or 20 µg/ml anti-human IgG/IgM (Jackson-Immunoresearch) in RPMI (Invitrogen) supplemented with 10% FCS (Bodinco). All stimulations were tested to be optimal for IL-10 production at the given concentrations (data not shown). After 48 h, supernatants were taken and B cells were restimulated with PMA (200 ng/ml), ionomycin (2 µg/ml), and LPS (100 ng/ml) for 6 h and the last 4 h in the presence of Brefeldin A (BrefA; 10 µg/ml; Sigma-Aldrich), followed by fixation with 1.9% PFA. Next, the cells were permeabilized 0.5% saponin buffer and stained for CD1d-PE (CD1d42, BD), CD5-PerCPeFluor710 (UCHT2, eBioscience), CD20-APC-eFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, Biolegend), CD27-APC (L128, BD), and CD38-FITC (HIT2, BD), IL-10-Biotin plus streptavidin-Qdot525 (JES3-12G8, Abd Serotec/invitrogen), TNF-α-eFluor450 (MAB11, eBioscience) and FcγR-binding inhibitor (eBioscience). FMOs were used for gate setting for all surface markers and cytokine expression.

B-T cell co-culture

1 x 10^5 B cells were stimulated with medium, ultra-pure LPS, CpG or anti-human IgG/IgM for two days, followed by removal of supernatant, washed, and co-cultured with autologous memory CD4^+ T cells (ratio 1:1) in the presence of 1 µg/ml endotoxin-free Derp1 (LoTox Derp 1, Indoor Biotechnologies) in RPMI supplemented with 10% FCS. CD4^+ T cells were first pre-incubated with blocking IL-10R antibodies (2 µg/ml; R&D Systems) or IgG1 isotype control (2 µg/ml) for 30 min at 37 °C. After six days, supernatants were taken and the cells were restimulated by PMA/ino and BrefA (as indicated above) and fixated with FoxP3 fixation/permeabilization buffer (eBioscience). The cells were stained for CD3-PerPCy5.5 (SK7, BD), CD20-Pacific Blue (2H7, Biolegend), CD25-PE (2A3, BD), IL-10-Biotin plus streptavidin-Qdot525 (JES3-12G8, Abd Serotec/invitrogen), TNF-α-PeCy7 (MAB11, eBioscience), FoxP3-FITC (PCH101, eBioscience), and FcγR-binding inhibitor (eBioscience). FMOs were used for gate setting for all surface markers and cytokine expression.

Cytokine Secretion assay, ELISA and Luminex

From PBMC, monocytes were first depleted prior to isolation of B cells using CD14 MicroBeads (Miltenyi Biotec). After the B-cell isolation, cells were stimulated for 3 days (adapted from Milovanovic et al. (25)). Next, the B cells were restimulated with PMA and ionomycin for 2 h, washed, and incubated for 1 hour with IL-10 catch reagent according the protocol (Miltenyi Biotec). After the incubation, cells were stained for IL-10-PE (dilution 1:25, Miltenyi Biotec), CD20-Pacific Blue (2H7, BioLegend), life death marker 7-AAD-PerCP (E00031-1632, eBioscience) and FcγR-binding inhibitor (eBioscience). Collected supernatants collected were measured by Enzyme-Linked Immunosorbent Assay (ELISA) for IL-10 (Sanquin) or by Luminex (IL-5, IFN-γ, IgG1, IgG2, IgG4, and IgE, Invitrogen/Biorad).
Western blot
For whole cell lysate, $1 \times 10^6$ B cells from 4 healthy donors from laboratory staff were stimulated for 5, 20, 60, 180 min with medium or LPS (100 ng/ml), washed and treated with a buffer containing 8% v/w glycerol, 3% SDS, and 100mM Tris-PO4 (EBSB) for 5 min. The lysate was heated to 95 °C for 5 min and then stored at -80 °C. The protein concentration of the whole cell lysates was determined by a bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Whole cell lysates (10 μg/lane) were boiled in SDS sample buffer (50 mM Tris-Cl (pH 6.8), 1% 2-ME, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) for 5 min before being subjected to electrophoresis on 10% or 12.5% SDS-PAGE gels. After separation, the proteins were transferred onto nitrocellulose (Millipore). The membrane was incubated with primary antibodies against STAT3, and Phospho-STAT3 (Tyr 705) (D3A7) (all from Cell signaling Technology) and then incubated with anti-rabbit IgG HRP conjugated secondary antibodies (Promega). The bands were visualized by an enhanced chemiluminescence reagent (Thermo scientific).

Statistical analysis
For statistical analysis, Kruskal-Wallis 1-way ANOVA and the Mann-Whitney U tests were performed. Differences in paired conditions were analyzed by Wilcoxon matched pairs test. Correlations were analyzed using SPSS Statistics 20 (IBM, New York, USA). Probability values less than 0.05 were considered significant.
Results

Patient characteristics
To study the function and phenotype of peripheral blood B cells, 13 non-smoking patients with AA (age, 19-44 years; 7 females and 6 males) and 13 age- and sex-matched non-smoking HC were included (age, 21-45 years). The patients with AA showed clinically controlled asthma without severe exacerbations for more than 6 months (GINA guidelines). They had the following characteristics (median): an ACQ value of 4, a FEV₁-% predicted of 101, a FEV₁/FVC (as measure for obstruction) of 0.80, FeNO of 30 and a PC₂₀-methacholine of 1.4 mg/ml (Table 1), which match values for mild to moderate bronchohyperreactivity. The HC showed no signs of asthma by an ACQ outcome of 0, a FEV₁-% predicted of 112, a FEV₁/FVC of 0.87, FeNO of 13 and a PC₂₀-methacholine of >19 mg/ml.

Positive SPT reactivity with a wheal larger than 5 mm was recorded against HDM in 12 patients with AA, against grass and/or tree pollen in 10 patients and against cat and/or dog in eight patients (Table 1). The median of total serum IgE levels was 108 IU/ml and the median for specific IgE for HDM was 13 IU/ml, for grass 0.7 IU/ml, for tree pollen 0.2 IU/ml, for cat 0.4 IU/ml and for dog 0.4 IU/ml (Table 1). The HC group showed a negative SPT for HDM, cat, dog, tree and grass pollen or Aspergillus and a median total serum IgE of 7 IU/ml was recorded, which was significantly lower than the patients with AA. The blood of patients and of their matched controls was processed and analyzed simultaneously. In addition, the lymphocyte count (PBMC/ml blood) and the frequencies of B and memory CD4⁺ T cells were similar between the groups (data not shown).

Lower numbers of CD24⁺CD27⁺ B cells in patients with AA
To investigate the frequency of the different Breg cell subsets, isolated peripheral blood CD19⁺ B cells were stained for CD1d, CD5, CD20, CD24, CD27 and CD38. Gating examples of a paired patient and healthy control are shown in Figure 1 to define the following three different subsets: CD1d⁺ B cells, CD24⁺CD38⁺ B cells and CD24⁺CD27⁺ B cells. No differences were seen in frequency of CD1d⁺ B cells (average of 2%; Fig. 1A) or in the smaller subset CD1d⁺CD5⁺ B cells (average of 0.3%; data not shown) between the groups. Of note, despite its confusing nomenclature the CD1d expression on human B cells is generally very low, but detectable as shown by figure 1. Although the percentage of transitional immature CD24⁺CD38⁺ Breg cells was significantly increased in patients with AA (Fig. 1B), the absolute numbers per ml blood were not similar to that described by Blair et al. in SLE patients (16). Interestingly, both the percentage and absolute numbers per ml blood of CD24⁺CD27⁺ B cells were significantly reduced in patients with AA compared to controls (Fig. 1C), while the distribution of naïve (CD27⁻) and memory B cells (CD27⁺) or CD24⁺ B cells were similar between the groups (data not shown). Of note, the frequency of CD24⁺CD27⁺ B cells was the highest of the Breg cell subsets (~27 % CD24⁺CD27⁺ vs. ~ 3 % CD24⁺CD38⁺ vs. ~2% CD1d⁺ B)}
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We did not detect any correlation between the percentage of CD24hiCD27+B cells or any of the other Breg cells and the level of total serum IgE, HDM-specific serum IgE, PC20, exhaled FeNO or medication usage in the asthmatic patients (data not shown).

Less intracellular IL-10 in B cell from patients with AA in response to LPS
One of the hallmarks of Breg cells is their capacity to produce IL-10. To compare the IL-10-producing capacity of B cells from AA and HC subjects, CD19+B cells were isolated from peripheral blood and stimulated for 2 days with medium alone, anti-IgG/IgM (general BCR trigger), LPS (TLR4 ligand) or CpG (TLR9 ligand). Subsequently, the cells were restimulated by PMA/ionomycin in the presence of Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>AA (n=13)</th>
<th>HC (n=13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (f)</strong></td>
<td>7 (13)</td>
<td>7 (13)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Age (yrs)</strong></td>
<td>26 (19-44)</td>
<td>24 (21-45)</td>
<td>0.9379</td>
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<tr>
<td><strong>BMI</strong></td>
<td>22 (19-35)</td>
<td>22 (18-24)</td>
<td>0.3517</td>
</tr>
<tr>
<td><strong>ICS/CT</strong></td>
<td>6(13) + 4(13)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>FeNO</strong></td>
<td>30 (13-80)</td>
<td>13 (5-21)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>ACQ</strong></td>
<td>4 (0-12)</td>
<td>0 (0-1)</td>
<td>-</td>
</tr>
<tr>
<td><strong>FEV1-%predicted</strong></td>
<td>101 (92-118)</td>
<td>112 (102-128)</td>
<td>0.0026</td>
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<tr>
<td><strong>FEV1/FVC</strong></td>
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<td>0.87 (0.77-0.97)</td>
<td>0.1637</td>
</tr>
<tr>
<td><strong>PC20-methacholine</strong></td>
<td>1.4 (0.3-9.6)</td>
<td>&gt;19</td>
<td>-</td>
</tr>
<tr>
<td><strong>SPT (&gt; 5mm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDM</td>
<td>12/13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Grass and/or Tree</td>
<td>10/13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cat and/or Dog</td>
<td>8/13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total IgE (IU/ml)</strong></td>
<td>108 (30-307)</td>
<td>7 (2-31)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Spec. IgE (IU/ml)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDM (n=9)</td>
<td>13 (0.11-50.6)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Grass (n=9)</td>
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<td>-</td>
</tr>
<tr>
<td>Tree (n=9)</td>
<td>0.2 (0.02-10.3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cat (n=9)</td>
<td>0.4 (0.01-12.4)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dog (n=9)</td>
<td>0.4 (0.1-2.7)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Allergic Asthma (AA); Healthy Controls (HC); female (f); years (yrs); Body mass Index (BMI); inhalation corticosteroids (ICS); combination therapy (CT); Exhaled nitric oxide (FeNO); Asthma Control Questionnaire (ACQ); Forcedexpiration volume in 1 second (FEV1); Forced vital capacity (FVC); provocative concentration of methacholine causing a 20% fall in FEV1 (PC20); Skin Prick Test (SPT); House dust mite (HDM)
of BrefeldinA to allow intracellular cytokine detection (Fig. 2A). In medium-conditioned B cells ~2 % IL-10 producers were found, while both BCR stimulation and LPS priming significantly further enhanced intracellular IL-10 levels by almost a two-fold (Fig. 2B). CpG showed the strongest IL-10 induction, inducing approximately 10%. Strikingly, only intracellular IL-10 in LPS-primed B cells of patients with AA was significantly lower compared to those from healthy controls (Fig. 2B), while equal IL-10 levels were found for medium-conditioned, BCR- and CpG-stimulated B cells. We analyzed the mean fluorescence intensity (MFI) values of the cytokine-expressing cells, demonstrating a similar MFI for IL-10+ cells in all conditions following background subtraction (MFI IL-10+ approx. 5200, data not shown) and similar between HC and patients with AA. This suggests that mostly the number of responding B cells varied per condition but not so much the quantity of IL-10 produced per B cell. No correlation was found between LPS-induced IL-10 levels and serum IgE levels (and HDM-specific IgE), PC20, exhaled
FeNO or medication use in patients with AA. The production of the more pro-inflammatory cytokine TNF-α was also analyzed to exclude general increased cytokine levels, but no differences were found between the two groups for any of the stimuli. The IL-10-producing B cells did not co-produce TNF-α (Fig. 2C).

Secreted IL-10 levels in the supernatant of CpG-stimulated B cells confirmed the flowcytometry data and showed clearly detectable IL-10 levels, but not different between HC (210 pg/ml) and AA donors (190 pg/ml, P = 0.47). Unfortunately, we were unable to detect secreted IL-10 or TNF-α by ELISA in the supernatant of stimulated B cells in response to LPS or anti-IgG/M, due to either limited cell numbers (a minimum of 3 x 10^5 B cells is required; data not shown) or high background levels (caused by anti-IgG/M itself; data not shown). Therefore, we validated the LPS-induced IL-10 production by conducting an IL-10 secretion assay allowing secretion and capture of IL-10 at the B cell surface. Using this assay, secreted IL-10 was studied in B cells from healthy laboratory donors stimulated with medium, LPS, anti-IgG/M or CpG for 3 days (adapted from Milovanovic et al. (25). A twofold increase of B-cell IL-10 was observed for both LPS and anti-IgG/M.

![Figure 2](image.png)

**Figure 2.** Intracellular IL-10 detection in total B cells. B cells were stimulated for 48 h by medium, lipopolysaccharide (LPS) (100 ng/ml), CpG (5 µg/ml) or anti-IgG/IgM (20 µg/ml) followed by restimulation with PMA/Ionomycin/LPS in the presence of BrefeldinA. (A) A representative gating example of IL-10 and TNF-α production upon LPS stimulation of a matched healthy control (HC) and allergic asthma (AA) donor. Fluorescence minus one (FMO) shows background levels of IL-10 and TNF-α. (B) Intracellular IL-10 and (C) intracellular TNF-α production were analyzed using flowcytometry.
stimulation, and an even higher increase for CpG, confirming the data obtained by intracellular cytokine staining described above (Appendix S1).

**Reduced LPS responsiveness of CD24<sup>hi</sup>CD27<sup>+</sup> B cells in allergic asthma patients**

We next questioned whether the lower LPS-induced IL-10 in B cells from patients with AA was the result of a selective impairment in one of the putative Breg subsets. Breg subset analysis showed that LPS stimulation of B cells strongly enhanced the number of IL-10<sup>+</sup> cells compared to medium in all three subsets, i.e. CD1d<sup>hi</sup> B cells (Fig. 3A), CD24<sup>hi</sup>CD38<sup>hi</sup> (Fig. 3B) and CD24<sup>hi</sup>CD27<sup>+</sup> B cells (Fig. 3C), although CD1d<sup>hi</sup> B cells tend to have a higher spontaneous production of IL-10 (~7%) in medium conditions. When comparing patients with AA and controls, we found less intracellular IL-10 for CD24<sup>hi</sup>CD27<sup>+</sup> B cells in patients with AA (Fig. 3C), but similar levels in the other two Breg subsets (Fig. 3A and 3B). In the remaining CD24<sup>+</sup>, CD27<sup>+</sup> or CD24<sup>-</sup>CD27<sup>-</sup> B cells, the IL-10 production was not significantly affected in patients with AA (Appendix S2A). Despite the fact that the different Breg cell subsets have been analyzed separately in the previous studies, they do have some overlap. Interestingly, a substantial part of the CD1d<sup>hi</sup> B cells (61.7% for HC and 56.1% for AA; *P* = 0.46) can be found within the CD24<sup>hi</sup>CD27<sup>+</sup> B cell subset and for both groups 17.5% of the CD24<sup>hi</sup>CD38<sup>hi</sup> are also CD24<sup>hi</sup>CD27<sup>+</sup> (*P* = 1.00). When CD1d<sup>hi</sup> B cells were removed from the analysis of the CD24<sup>hi</sup>CD27<sup>+</sup> population, the decreased IL-10 production still remained in the B cells from patients with AA. Additionally, the exclusion of the CD24<sup>hi</sup>CD38<sup>hi</sup> B cells gave similar results (Appendix S2B). Altogether, these data suggest a diminished IL-10 response of CD24<sup>hi</sup>CD27<sup>+</sup> B cells from patients with AA compared to controls upon LPS stimulation.

**TLR4 expression on B cells from patients with AA compared to healthy controls**

To investigate whether the differences in B cell IL-10 production between the groups were a consequence from differences in TLR4 expression in B cells, we

![Figure 3](image-url)

**Figure 3.** Intracellular IL-10 in various B cell subsets in response to lipopolysaccharide (LPS). B cells were stimulated by medium or LPS and treated as described in Fig. 2. Intracellular IL-10 in CD1d<sup>hi</sup> B cells (A), in CD24<sup>hi</sup>CD38<sup>hi</sup> B cells (B) and in CD24<sup>hi</sup>CD27<sup>+</sup> B cells (C) were analyzed using flowcytometry.
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evaluated the TLR4 levels by flowcytometry. Although the expression of TLR4 on B cells is rather low compared to monocytes (data not shown, MFI of 3629 (HC) and 3280 (AA), \(P = 0.10\)), a significant higher MFI was detected both for HC donors and patients with AA compared to total T cells (as a negative control) (Fig. 4A). Nevertheless, we did observe a trend towards a decreased TLR4 expression in AA patients, suggesting that their lower IL-10 response to LPS might be due to lower TLR4 expression, however, this was not attributed to either one of the B cell subsets (Fig. 4B). Interestingly, we detected the highest TLR4 expression on the

Figure 4. (A) The geometric mean fluorescence intensity of TLR4 on B cells was measured on PFA-fixed PBMCs from healthy controls (HC) and allergic asthma (AA) donors, using the TLR4 expression on total T cells as negative control. (B) TLR4 expression on the different Breg subsets. (C) 1 x 10⁶ B cells from healthy laboratory donors were stimulated with medium or LPS (100 ng/ml) and incubated for 5, 20, 60 and 180 min. At the indicated time points, cells were harvested and nuclear extracts were prepared. Equal concentrations of protein were subjected to electrophoresis and blots were reacted with antibodies specific to phospho-Tyr705 of STAT3 and total STAT3, followed by development using enhanced chemiluminescence. One representative blot out of \(n = 3-4\) with similar results is shown.
CD1d<sup>hi</sup>B cells, following by the CD24<sup>hi</sup>CD27<sup>+</sup> B cells and very low expression on the CD24<sup>hi</sup>CD38<sup>hi</sup> B cells, which correlated well with their capacity to produce IL-10 in response to LPS (Fig. 4B).

Furthermore, to confirm LPS-induced activation of B cells, western blots were performed using whole-cell extracts from primary B cells of healthy labdonors stimulated with LPS or medium only. We focused on signal transducer and activator of transcription 3 (STAT3) signaling, as in previous studies it was shown that LPS-induced phosphorylation of STAT3 was implicated in IL-10 production in monocytes (26;27) and murine Breg cells (28). Here, we also detected strong induction of STAT3 phosphorylation after 180 min of exposure to LPS compared to medium (Fig. 4C), confirming that at least in healthy labdonors LPS induced the STAT3 signaling pathway in B cells, eventually leading to IL-10 production. Unfortunately, due to limited cell numbers we could not study the LPS-induced STAT3 phosphorylation in patients with AA.

**LPS-primed B cells in patients with AA induce less IL-10-producing T cells**

To study whether the lower frequency of IL-10-producing B cells from patients with AA has any functional consequences for allergen-specific T-cell cytokine responses, co-cultures were set-up with primed B cells and autologous CD4<sup>+</sup> memory T cells. These cells were co-cultured for 6 days in the presence of house...
dust mite allergen DerP1 to evaluate whether primed B cells could influence the cytokine profile of allergen-specific T cells. The induction of anti-inflammatory responses such as CD4^+CD25^hiFoxP3^+ Treg cells or IL-10^+ CD4^+CD25^hiFoxP3^+ Treg cells was not different between the two groups (data not shown). Nevertheless, the intracellular IL-10 production by CD4^+ T cells (putative Tr1 cells) was enhanced in the presence of CpG or LPS-primed B cells. Interestingly, in patients with AA, this induction was only observed in the condition with BCR- or CpG-stimulated B cells, but not with LPS-primed B cells, suggesting a specific impairment in the case of LPS stimulation (Fig. 5A), (Appendix S3). Remarkably, despite the higher IL-10 secretion in CpG-stimulated B cells, LPS-stimulated B cells from HC induced at least equal or even more IL-10^+ T cells. The involvement of B cell derived IL-10 in the putative Tr1 cell induction was confirmed both in the CPG and the LPS condition of HC where a pre-incubation of the CD4^+ memory T cells with blocking IL-10R antibodies reduced T cell IL-10 production (Appendix S3). This was also observed in the condition of CpG-primed B cells of patients with AA, but not for the LPS-primed B cells (Appendix S3). Interestingly, blocking T cell IL-10 receptor in LPS-primed conditions substantially reduces the IL-10^+ T cell induction in control subjects though not fully to levels found in medium conditions, suggesting that for

![Figure 6](image.png)

**Figure 6.** (A) B cells were sorted into CD24^hiCD27^+ B cells and remaining cells. (B) 1 x 10^5 CD24^hiCD27^+ B cells or the remaining cells were stimulated with medium or ultra-pure LPS for two days, followed by removal of supernatant, washed, and co-cultured with autologous memory CD4^+ T cells (ratio 1:1) in the presence of 1 μg/ml endotoxin-free Derp1. CD4^+ T cells were first pre-incubated with blocking IL-10R antibodies (2 μg/ml) or IgG1 isotype control (2 μg/ml) for 30 min at 37 °C. After 6 days, the cells were restimulated, fixed and staining for IL-10 using flowcytometry. The T cells were gated for CD3^+FoxP3^-TNFα.
LPS-induced B cells also other factors than IL-10 may be involved in subsequent T cell IL-10 production (Fig. 5A).

Next to IL-10, intracellular cytokine production of TNF-α and IFN-γ showed similar number of these cells in both groups and IL-4 was hardly detectable after culturing with LPS-primed B cells (data not shown). However, a higher Th2/Th1 ratio (IL-5/IFN-γ) was found in the supernatant of B and T cell co-cultures from patients with AA compared to controls (Fig. 5B). Additionally, significantly more IgG1 and a trend for IgE production were detected in cultures of LPS-primed B cells and T cells from patients with AA compared to those from controls (Appendix S4), while IgG2 and IgG3 did not change and IgG4 levels were very low in both groups (data not shown) suggesting a more pro-inflammatory type 2 profile in patients with AA both with respect to cytokines and immunoglobulins.

**CD24^{hi}CD27^{+} B cells are essential for the induction of IL-10^{+} T cells**

We next assessed whether the induction of IL-10-producing T cells could be attributed to the CD24^{hi}CD27^{+} B cell population (Fig. 6). To address this question, 10 HC subjects donated blood for a second time and B cells were sorted by flowcytometry into two populations, CD24^{hi}CD27^{+} B cells and the remaining cells (CD24^{low}CD27^{+} and CD24^{low}CD27^{-} B cells) (Fig. 6A). Next, the cells were primed with medium or LPS for 2 days, followed by a co-culture with CD4^{+} memory T cells, as stated above. Interestingly, the sorted CD24^{hi}CD27^{+} B cells shower similar inducing capacity as the total B cells, whereas the remaining B cells could not induce IL-10^{+} T cells after LPS priming (Fig. 6B). Blocking the IL-10 receptor on T cells significantly reduced the levels of IL-10 production to medium levels, confirming the exclusive role of IL-10-producing CD24^{hi}CD27^{+} B cells in the induction of IL-10^{+} T cells and its dependence on B-cell-derived IL-10.
Discussion

Regulatory B cells have been identified that have an impaired function in autoimmunity. Here, we demonstrated that B cells from patients with allergic asthma also have a lower capacity to produce IL-10 in response to LPS compared to B cells from controls. The CD24<sup>hi</sup>CD27<sup>+</sup> B cell subset seems to be responsible for this effect. The expression of the pro-inflammatory cytokine TNF-α was similar in total B cells and the subsets, indicating that primarily the IL-10 response was specifically altered in B cells from patients with AA. Furthermore, this difference translates in a functional effect, namely the ability of CD24<sup>hi</sup>CD27<sup>+</sup> B cells to induce IL-10<sup>+</sup> T cells and, therefore, may point at a role for a weakened Breg function in patients with AA.

We have compared the frequency of different Breg cell membrane markers on peripheral blood B cells based on reports describing functional Breg cells in humans, i.e. CD1d<sup>hi</sup> B cells (14;19), CD24<sup>hi</sup>CD27<sup>+</sup> B cells (17) and CD24<sup>hi</sup>CD38<sup>hi</sup> B cells (16). Blair et al. observed an increased frequency of CD24<sup>hi</sup>CD38<sup>hi</sup> B cells in SLE patients, but with an impaired capacity to inhibit IFN-γ production in stimulated T cells. Intracellular IL-10 production in this particular B cell subset were not studied (16). Interestingly, we also observed an increase in CD24<sup>hi</sup>CD38<sup>hi</sup> B cells in patients with AA, but with similar IL-10 frequencies between the groups. However, as we did not study the ability of isolated CD24<sup>hi</sup>CD38<sup>hi</sup> B cells to inhibit cytokine secretion by T cells, we cannot exclude functional differences for this B cell subset in allergic asthma, despite equal intracellular IL-10 frequencies.

The regulatory capacity of CD24<sup>hi</sup>CD27<sup>+</sup> B cells was demonstrated by Iwata et al. by showing the ability of this B cell subset to reduce cytokine production by monocytes (17). Interestingly, this reduction was not IL-10-dependent and these cells could not reduce cytokine production by T cells. Here, we observed a lower LPS-induced IL-10 production by CD24<sup>hi</sup>CD27<sup>+</sup> B cells in patients with AA versus controls, which upon further co-cultures with T cells, was accompanied by a reduced IL-10 expression in T cells. Although we only studied single sorted subsets in HC subjects, but given the lower IL-10 production in CD24<sup>hi</sup>CD27<sup>+</sup> B cells in response to LPS in patients with AA, it is tempting to speculate that this subset might be responsible for the lower induction of IL-10-producing T cells observed in the co-cultures with total B cells.

It was shown in several studies conducted with traditional European farmer families, that growing up on farms protects against the development of allergy and asthma and that both bacterial and fungal exposures may be important (4-6). Indeed, exposure to bacterial components, such as endotoxin (LPS) and muramic acid (component of peptidoglycan), were inversely related to allergic sensitization, childhood asthma or wheezing (29;30). This also accounts for microbial products found in floor dust from urban living rooms (31-33). Not only the level of microbial content but also the extent of diversity was important for the inverse relationship with allergies and asthma (33-35). Although there
is a positive correlation between the levels of bacterial DNA and the levels of endotoxin found in farm dust (36), suggesting that CpG and endotoxin will both be present to a similar extent in daily life, it is unclear whether this correlation can also be attributed to ‘poor’ microbe environments like urban homes. It may be questioned whether the in vitro stimulation conditions of B cells we have applied here are realistic to in vivo exposure in daily life and whether the presence of CpG may compensate for the defect in LPS-induced IL-10 production of B cells from patients with allergic asthma. Despite this assumption, it is unclear what the net effect is when CpG and LPS would act together on B cells, and whether a low concentration of CpG would still induce a similar B cell IL-10 production in both groups or that, like the weak B-cell activator LPS, it would also show a weaker response in allergic patients. Interestingly, it was showed that ‘farm’ bacterial DNA potentiated LPS-induced cytokine production in PBMC, while ‘urban’ bacterial DNA did not (36), suggesting that either the activity or the content of bacterial DNA versus endotoxin in dust is different between rich and poor microbe environments resulting in a different net effect on single cells and keeping open the option that the LPS conditions described in this paper may occur in vivo.

Also on the level of the innate receptors involved in the recognition of these bacterial components (CD14, TLR2 and TLR4), mRNA expression was shown to be increased on PBMC from farmer’s children and in particular exposure of pregnant mothers to stables was positively correlated with increased TLR expression (37;38). Although TLR4 expression on B cells is relatively low compared to TLR2 and TLR9, B cells are clearly able to respond to LPS (39-41). Furthermore, inflammatory conditions were associated with an upregulation of TLR4 expression on B cells (41-44). In our study, we confirmed that although TLR4 expression on B cells is quite low, LPS was capable of inducing STAT3 signaling, confirming its capacity to activate human B cells of healthy controls. Because the phosphorylation of STAT3 was only significantly increased after 180 min, we cannot rule out that this effect is not a direct consequence of TLR4 signaling but alternatively the result of a secondary stimulation via other factors initially induced by LPS, but altogether still induced by LPS stimulation only. Although there was a trend in reduced TLR4 levels in patients with AA compared to HC subjects (but not in the individual Breg subsets), we cannot exclude that the lower LPS-induced IL-10 expression in B cells of patients with AA may as well be explained by differences in TLR4-induced signaling pathways, as previously suggested for monocytes from allergic patients, which showed an impaired phosphorylation of MAPK pathways upon LPS stimulation (45).

While there is clear evidence in different animal models that enhanced activity of IL-10-producing Breg cells strongly suppresses allergic inflammation (15), there is only one study available in food allergy, studying IL-10 expression in B cells in patients suffering from food allergy. IL-10-expressing B cells were downregulated in response to milk antigens in PBMC from allergic, but not from healthy individuals (46). It is also still unclear whether the IL-10-producing Breg cells in the circulation are the ones that will migrate to the target organs, such
as the lung, to act in a protective way or that maybe other local Breg cell subsets are responsible for this. In contrast, evidence for a protective role in allergy and asthma of IL-10-producing T cells or FoxP3⁺ Treg cells is extensively reported in both animal models and human studies (7). In addition, IL-10 production plays a central role in beneficial effects of immunotherapy (47). In this study, we demonstrated a causal link between IL-10-producing B cell and IL-10⁺ T cells, suggesting that B cell-derived IL-10 can instruct T cells for their regulatory capacity. Indeed, several animal studies have demonstrated that part of the effect of IL-10-producing B cells is mediated by induction of IL-10-producing T cells or FoxP3⁺ Treg cells (10;15). These findings imply that targeting for regulatory B cells as a novel therapeutic approach may embrace two regulatory systems simultaneously, i.e. regulatory B cells and IL-10-producing regulatory T cells. New studies have to find out whether this may lead to a much more efficient suppression of allergen-specific immune responses in patients with AA.

In conclusion, further research should focus on the identification of pathways and molecules that enhance the number and/or activity of Breg cells, in which the CD24⁺CD27⁺ B cell subset may be interesting to be targeted with respect to the treatment of allergic asthma. Those molecules will be interesting candidates to include in current immunotherapy protocols and improve their efficacy in establishing longer lasting protection against allergic asthma.

Conflict of interest
This project was supported by Netherlands Asthma Foundation (NAF grant 3.2.10.072). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare no conflict of interest.

Acknowledgements
We thank K. Jansen for invaluable help in the recruitment and selection of patients and controls, A.C. van der Linden for performing the blood draws and G. van der Zon for the western blots. We thank all the blood donors who participated in this study.
References


Weaker regulatory B-cell function in allergic asthma


Supplementary figures

Figure S1. B cells (3x10^5) from healthy laboratory donors were stimulated for 72 h with anti-IgG/M (20 µg/ml), LPS (100 ng/ml), CpG (5 µg/ml) and medium. Medium values (~1.5%) were subtracted from the stimulated conditions for each individual. IL-10 secretion assay (Miltenyi Biotec) was performed and shows the percentage of IL-10-producing B cells. N=3.

Figure S2. Intracellular IL-10 (A) in CD24<low>CD27<−>, CD24<+> or CD27<+> B cells. (B) The expression of IL-10 in CD24<+>CD27<+> B cells excluded for CD1d<hi> and CD24<+>CD38<hi> B cells. The B cells were treated as described in Figure 2.
Figure S3. Co-culture of BCR- or CpG-stimulated B cells and T cells. B cells were treated as described in Figure 2, followed by a 6 day 1:1 co-culture with autologous CD4⁺ T cells CD4⁺ T cells (pre-incubated with isotype or anti-IL-10R antibodies; 2 µg/ml), and DerP1 (1 µg/ml). Cells were restimulated to determine intracellular cytokines by flowcytometry. Percentage in IL-10⁺ CD4⁺ T cells was calculated. One pair was excluded as the patient with AA was not allergic for HDM (n=12).

Figure S4. Immunoglobulin production of LPS-primed B cells in co-culture with T cells. Co-cultures were setup as described in Figure 4. After 6 days, supernatants were harvested and immunoglobulins (IgG1, IgG2, IgG4, and IgE) were measured by immunoglobuline isotyping assay (Biorad-luminex). Background levels of medium-primed and LPS-primed B cells are shown.