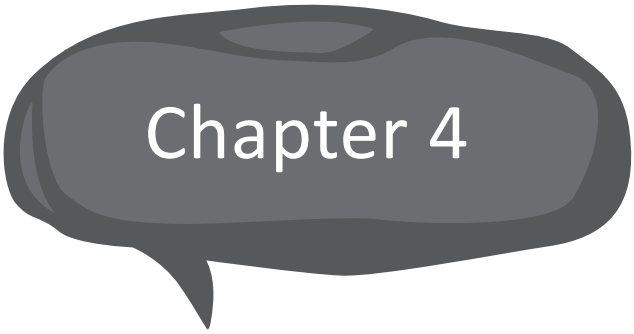

Toll-like receptor ligation for the induction of regulatory B cells

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Abstract

Toll-like receptors (TLRs) are key components for the recognition of micro-organisms, the initiation of innate immunity and for promoting adaptive immune responses. TLR signaling in B cells, in addition to B cell receptor or CD40 ligation, plays an important role in B cell differentiation and activation. In contrast, various infectious agents and/or TLR ligands can also prime B cells to induce tolerance and downregulate inflammatory reactions; those B cells are called regulatory B (Breg) cells and are characterized by a dominant IL-10 production. Several studies have suggested that Breg cells are impaired in patients with autoimmune diseases and allergic asthma. However, the role for TLR ligands in the induction of Breg cells as a potential therapy for some of these inflammatory diseases has not yet been investigated. Here, we provide detailed instructions how to analyze and validate cytokine production in human and mouse B cells in response to various TLR ligands. Furthermore, we describe an assay to investigate the suppressive properties of TLR-induced B cells to confirm their regulatory B cell status.

1. Introduction

Toll-like receptors (TLRs) represent one of the most studied classes of pattern recognition receptors and are key components for the recognition of micro-organisms and activation of immune cells. TLR ligands have become of interest in B cell research because of their application as potent adjuvants in novel vaccine strategies. However, several reports have been published over the past years highlighting a role for TLRs in enhancing anti-inflammatory responses instead, possibly promoting the development or activation of regulatory B (Breg) cells. Several mouse studies have demonstrated a role for signaling via TLR2, TLR4, and TLR9, either or in combination with CD40 engagement, CD80/CD86 or B cell-activating factor (BAFF), to induce optimal IL-10 production by B cells with regulatory capacities (1-7). For example, during the bacterial infection by *Salmonella typhimurium*, MyD88-signaling in B cells suppressed protective immunity via IL-10, affecting natural killer cells, neutrophils, and effector T cells. Prolonged survival upon infection was displayed in mice with a B-cell specific deficiency in both TLR2 and TLR4, suggesting that the B-cell-mediated suppression resulted from direct microbial sensing by the B cells (8). Furthermore, ligation of TLR2 by ligands from *Helicobacter felis* induced IL-10-producing B cells in a MyD88-dependent manner, which suppressed helicobacter-induced pathology not through the direct production of IL-10 but via the generation of suppressive IL-10 secreting Tr1-like cells (9). Other bacterial antigens, such as the TLR4 ligand lipopolysaccharides (LPS) and the TLR9 ligand CpG oligonucleotides (ODN) were also described as strong inducers of IL-10 expression in murine B cells (10;11). Interestingly, adoptive transfer of LPS-activated B cells reduced the incidence of spontaneous type 1 diabetes for about one year in prediabetic NOD mice (12). Next to bacterial, also certain parasite-derived molecules, such as extracts from *Leishmania major* and *Brugia malayi* or milk-derived sugars comparable to the ones found in soluble egg antigens (SEA) from *Schistosoma mansoni*, induced IL-10-producing B cells most likely via TLR4 (13-15). In addition, CpG treatment of mice ameliorated spontaneous type 1 diabetes (16) and allergic conjunctivitis by TLR9 ligation and induction of IL-10 in B cells (17). These mouse studies show an important role for TLR2, TLR4 and TLR9 ligation in the development of Breg cells and highlight its potential to boost Breg cell development for treatment of various inflammatory diseases.

Human IL-10-producing Breg cells share phenotypic and functional characteristics with the murine counterpart and have been studied primarily in allergic and autoimmune diseases, where they were shown to be functionally impaired in their IL-10 response or regulatory activity (18-23). In some of the aforementioned cases, Breg cell defects also account for B cell IL-10 responses to TLR ligands. For example, CpG induced less IL-10 in B cells from patients affected by multiple sclerosis (MS) compared to healthy controls (24), which was also confirmed in autoimmune thrombocytopenia (25). Whereas TLR4 stimulation is

very potent on mouse B cells, human B cells produce rather modest IL-10 levels after exposure to LPS *in vitro* due to a relatively low TLR4 expression compared to TLR2 and TLR9 at least in healthy individuals (20;23;26-28). In several inflammatory conditions an upregulated TLR4 expression on B cells was described, suggesting a role of TLR4 activated B cells during infection (29-32). In the context of another inflammatory disease, i.e. allergic asthma, we showed that LPS-primed IL-10 from B cells of allergic asthma patients was reduced compared to that of healthy individuals and the subsequent induction of IL-10-producing T cells *in vitro* was abrogated as well (23). The impaired LPS response of B cells in allergic asthma patients may therefore play a role in the pathophysiology of asthma.

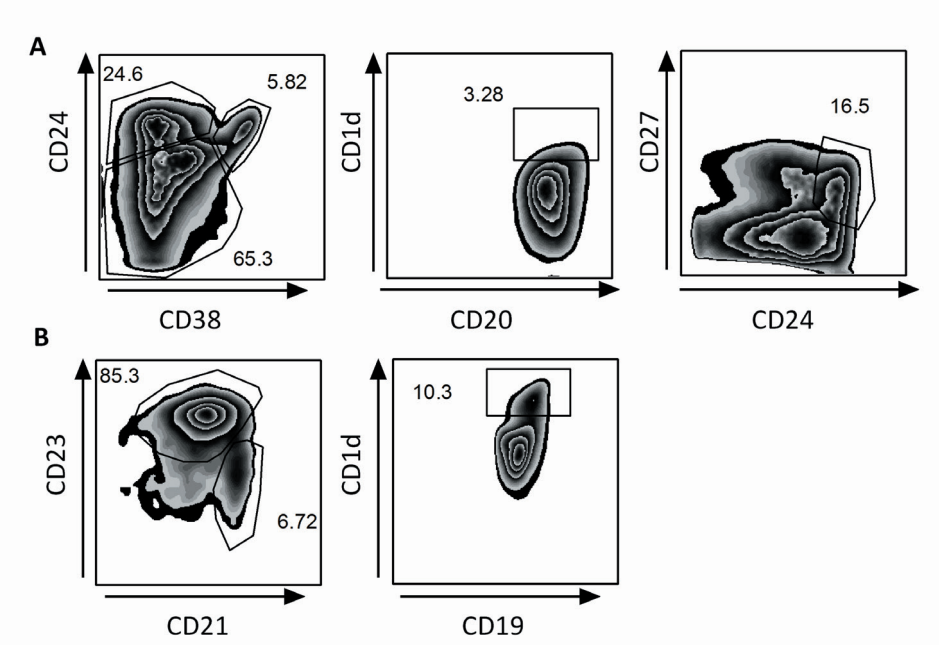


Figure 1. Main phenotypic markers of human and murine Breg cells. (A) Breg cells from human peripheral blood are $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD1d^{hi}$ (pre-gated on $CD20^{+}$ cells). (B) Murine Breg cells are $CD21^{hi}CD23^{low}$ and $CD1d^{hi}$ (pre-gated on $CD19^{+}$ splenocytes).

As outlined above, Breg cell function in response to some TLR ligands seem to be reduced in human autoimmune and allergic diseases. In contrast, recent reports suggest that the development and activity of Breg cells is increased in microbial infections, as shown in patients having chronic infections with hepatitis B virus (33), *Mycobacterium tuberculosis* (34) or the helminth *Schistosoma haematobium* (22). Although a direct role for TLR-dependent generation of Breg cells in these infections has not yet been established, TLR activation by various pathogen-derived ligands such as the TLR1/2 agonist Pam3CysK, a TLR2 ligand in SEA, and the TLR2/6 agonist Pam2CGDPKHPKSF were shown to activate IL-10 production in human B cells (20;35;36).

Various distinct and overlapping Breg subsets have been described in mice and humans of which the respective roles in response to TLRs can be investigated. For example, CD1d expressing B cells are described in both species (22), while $CD24^{hi}CD38^{hi}$, $CD24^{hi}CD27^{+}$ and $CD25^{hi}$ and $CD71^{hi}$ expression has been attributed to human Breg cells only (19;20;37) (as depicted in Fig. 1A) and $CD5^{+}$, $CD21^{hi}CD23^{hi}$ or $CD21^{hi}CD23^{low}$ were shown to be markers expressed by mouse Breg cells (Fig. 1B). However, as some of these markers are overlapping or these populations still contain non-IL-10-producing B cells, a unique set of markers or transcription factors that exclusively identifies Breg cells does not exist. Whether or not all B cells can develop suppressive functions following TLR activation is still under debate: Mizoguchi *et al.* proposed that only certain 'innate' B cell subsets will develop into Breg cells following TLR ligation (38), while others have suggested that all immature progenitor cells have the potential to progress into mature IL-10-producing B cells after ligation by TLRs and CD40 directly (39). Yet another hypothesis has been presented by Fillatreau and co-workers, which proposed that B cells can acquire only suppressive functions after a stepwise activation process initiated by TLR ligands and followed by BCR and CD40 reinforcement (40).

Most studies investigating the presence and function of Breg cells following TLR stimulation limit their analysis to IL-10 as the major anti-inflammatory and effector cytokine of Breg cells. However, it is often neglected that, next to IL-10, TLR ligation can simultaneously contribute to the development of inflammatory responses (10;35). For example, CpG, which is a strong IL-10 inducer, leads to strong B cell activation, proliferation, immunoglobulin production and expression of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (41-43). Indeed, elevated numbers of IL-6-producing B cells were observed in MS patients after *in vitro* ligation with CD40 and B-cell receptor with CpG, which was demonstrated to have a pathogenic role in the development of chronic experimental autoimmune encephalomyelitis in mice (44). Because of the recent interest to study the TLR-induced generation of Breg cells, it is crucial to use optimized assays for the phenotypic characterization of Breg cells and the analysis of both inflammatory and anti-inflammatory responses. Flow cytometry and enzyme-linked immunosorbent assay (ELISA) allow the detection of cytokines such as IL-10. However, while the qualitative IL-10 contribution of each individual Breg subset can easily be monitored by flow cytometry, ELISA provides more quantitative information, but requires a preceding cell sorting of the subsets before stimulation.

Although high IL-10 secretion is regarded as being anti-inflammatory and a marker for Breg cells, it is possible that the TLR-primed B cells did not acquire any immune suppressive activity or act regulatory in an IL-10-independent manner. Therefore, it has a strong additive value to investigate whether TLR-primed B cells show any suppressive capacity on the effector function of other cells either *in vivo* or *in vitro*. Indeed various studies have shown that Breg cells can influence several aspects of innate and adaptive immunity and either one

of these or all targets should be included in the study to the development of TLR-induced Breg cells: (1) monocyte-induced inflammatory responses are suppressed in their release of pro-inflammatory cytokines (20); (2) the Th1/ Th2 balance is manipulated by inhibition of antigen-specific T cell proliferation in both Th1 and Th2 skewed diseases; and (3) regulatory responses are potentiated by the induction and/or recruitment of IL-10-producing T cells and $FoxP3^+$ Treg cells (19;21-23).

In conclusion, various TLR ligands can be used as potent B effector or Breg cell inducers *in vitro*. However, to be able to get a better insight in the acquired regulatory capacity, a number of parameters needs to be considered, that is (1) the culture conditions (cell numbers per well, duration of stimulation, concentration of TLR ligands), (2) the method of cytokine detection (flow cytometry and ELISA, mimicking T cell interaction or not, see Fig. 2 and 3), (3) the ratio of produced anti-inflammatory versus pro-inflammatory cytokines (IL-10 versus IL-6, Fig. 2 and 3), and (4) a co-culture with other immune cells to evaluate their suppressive capacity on the effector function of other immune cells (see Fig. 4).

2. Materials

2.1 Isolation of B and T cells

1. Lysis buffer: 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, in MilliQ, sterile.
2. MACS buffer: 0.5% BSA, 2 mM EDTA in PBS, sterile.
3. MACS Midi magnets and LS columns (Miltenyi Biotec).
4. Anti-mouse and anti-human CD19 MicroBeads (Miltenyi Biotec). (see Notes 1 and 2)
5. Anti-mouse and anti-human memory $CD4^+$ isolation MicroBeads (Miltenyi Biotec).
6. 100 µm cell strainers used for mashing the spleen in 50 ml tubes (Greiner Bio-One.
7. Ficoll/Hypaque.

2.2 Stimulation of B cells

1. Mouse: RPMI 1640 + Glutamax (Invitrogen Life Technologies) supplemented with 5% heat-inactivated FCS (Greiner Bio-One), 5×10^{-5} M 2-Mercaptoethanol, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin).
2. Human: RPMI 1640 supplemented with 10% heat-inactivated FCS (Greiner Bio-One), 1 mM pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.
3. 96-well round bottom plates (Greiner Bio-One).
4. TLR ligands (stock concentrations): CpG2006 (1 mg/ml, Invitrogen), CpG1826 (1 mg/ml, Invitrogen), Ultrapure LPS (2.5 mg/ml, Invitrogen), FSL-1 (1 mg/ml, EMC microcollection), PGN (1 mg/ml, Sigma-Aldrich) and Pam3-Cys (1 mg/ml, EMC microcollection); store in aliquots at -20 °C.
5. 0.1 mg/ml Phorbol 12-myristate 13-acetate (PMA), 0.5 mg/ml ionomycin and 5 mg/ml Brefeldin A; store in aliquots at -20 °C.

2.3.1 Quantification of cytokine production by flow cytometry

1. 37% paraformaldehyde.
2. 96-well V-bottom plates (Greiner Bio-One).
3. FACS buffer: 0.5% BSA, 2 mM EDTA in PBS, non-sterile.
4. Permeabilization buffer: FACS buffer supplemented with 0.5% saponin.
5. Flow cytometer.
6. Mouse antibodies for flow cytometry: anti-mouse CD1d-PE (1B1, ebioscience), anti-mouse CD19-APC-Cy7 (1D3, BD Pharmingen), anti-mouse CD21-APC (B3B4, ebioscience), anti-mouse CD23-FITC (7G6, BD Pharmingen), anti-mouse IL-10-PE (JES5-16E3, eBioscience), and anti-mouse FcγRII/III (2.4G2, Bioceros).
7. Live-dead fixable Aqua dead cell staining kit (Invitrogen).
8. Human antibodies for flow cytometry: CD1d-PE (51.1, eBioscience), CD20-APCeFluor780 (2H7, eBioscience), CD24-PeCy7 (ML5, ITK Diagnostics),

CD27-APC (L128, BD), CD38-FITC (HIT2, BD), IL-10-biotin (JES3-12G8, Abd Serotec) with streptavidin-Qdot525 (Invitrogen), TNF-α-eFluor450 (MAB11, eBioscience) and FcγR-binding inhibitor (eBioscience) (see Note 10).

2.3.2 Quantification of cytokine production by ELISA

1. BD OptEIA mouse IL-10 and IL-6 ELISA sets (BD biosciences).
2. Human IL-10 and IL-6 ELISA sets (Sanquin).

2.4 Detection of Tr1/FoxP3 T cells by flow cytometry after co-culture

1. FoxP3 permeabilization/fixation buffer (eBioscience).
2. Human antibodies: CD3-PerCPeFluor710 (OKT-03, eBiosience), CD20-APCeFluor780 (2H7, eBioscience), CD25-PE (2A3, BD), IL-10-biotin with streptavidin-Qdot525, TNF-α-PeCy7 (MAB11, eBioscience), FoxP3-eFluor450 (PCH101, eBioscience), and FcγR-binding inhibitor (eBioscience).

Figure 2. IL-10 and IL-6 expression of human B cells in response to TLR stimulation. 3 x 10^5 isolated peripheral blood $CD19^+$ B cells from healthy donors were stimulated with the TLR ligands LPS (100 ng/ml), Pam3-Cys (5 μg/ml), FSL-1 (5 μg/ml), and PGN (5 μg/ml), or cultured in medium as a negative control. (A) The frequency of $IL\text{-}10^+$ B cells was determined after 2 days stimulation followed by 6 h culture with PMA/ionomycin (BrefeldinA added in the last 4 h) *n* =6. (B) Secreted IL-10 measured by ELISA after 2 days stimulation with TLR ligands *n* =6-12. (C) IL-10 secretion in the supernatant of B cells cultured with TLR-2 ligands for 2 days (no restimulation) or restimulated with PMA/ Ionomycin/ BrefA (PIB) for 6 h. The cytokine production before the Golgi stop resembles the flow cytometry data (Fig. 2A). In addition, the cells were cultured after TLR priming with CD40L expressing cell line (J558) to mimicking B and T cell interaction for an additional 24 h. This figure represents 3 independent experiments. (D) IL-6 secretion after 2 days of stimulation measured by ELISA *n* =6.

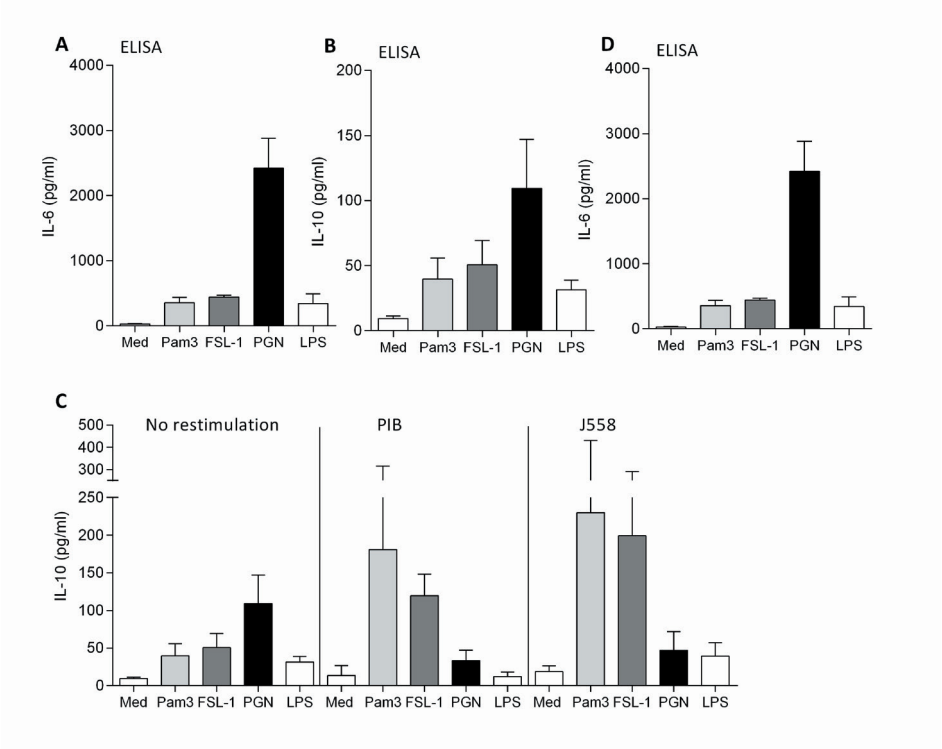
3. Methods

3.1.1 Isolation of mouse CD19$^+$ B cells

1. Single cell suspensions are prepared from mouse spleens using 100 µm cell strainers.
2. Erythrocytes are lysed from the spleen suspension using 2 minutes incubation with lysis buffer.
3. Cells are washed once with MACS buffer and are incubated with anti-mouse CD19 magnetic beads in MACS buffer and CD19$^+$ B cells are positively selected on LS columns according to the manufacturer's instructions (see Notes 1 and 2).

3.1.2 Isolation of human CD19$^+$ B and T cells

1. PBMC are isolated from blood of healthy donors (after their informed consent and in accordance with protocols approved by the local authorities) by density gradient centrifugation over Ficoll/Hypaque.
2. Cells are incubated with anti-CD19 magnetic beads (Miltenyi Biotech) in MACS buffer and positive selection of CD19$^+$ cells on LS/MS columns according to the manufacturer's instructions (see Note 3).
3. Flow through cells are collected to isolate CD4$^+$ memory T cells according to the manufacturer's instructions.



3.2 Stimulation of B cells with TLR ligands

1. MACS isolated B cells are washed twice with complete RPMI.
2. A minimum of 3.0 x 10^6 B cells/ml are needed to evaluate secreted cytokine levels in response to weak TLR ligands (see Note 4).
3. TLR ligands are diluted in complete RPMI two-fold concentrated, yielding the following final concentrations after addition of cells. Mouse: CpG1826 5 µg/mL; LPS 100 ng/mL; FSL-1 10 µg/mL; Pam3-Cys 10 µg/mL; PGN 10 µg/mL. Human: CpG2006 1 µg/ml; LPS 100 ng/ml, FSL-1 5 µg/ml; Pam3-Cys 5µg/ml; PGN 5µg/ml.
4. 100 µl of two-fold concentrated TLR ligands are added per well of a 96-well round bottom plate (Greiner Bio-One). Next 100 µl B cells (3x10^5) are added per well (total volume of 200 µl).
5. Murine cells are incubated at 37°C, 5% CO_2 and humidified atmosphere for three days (for flow cytometric IL-10 analysis, see Note 6) or five days (for ELISAs, see Notes 5 and 7). Human cells are incubated for two days (see Notes 5 and 7).

3.3.1 Quantification of cytokine expression by flow cytometry

Two different techniques can be applied for cytokine detection in TLR-activated B cells, however it is important to take into account that intracellular cytokine visualization after TLR ligation by flow cytometry needs an additional restimulation, such as PMA/ ionomycin/ BrefeldinA (PIB), which is not applied for the analysis of secreted cytokine levels in the supernatant by ELISA (see section 3.3.2). As a consequence the data of the two assays may not be fully comparable and the difference in assay processing should be carefully taken into account while drawing conclusions. This is highlighted and explained in an example where we compared different TLR2 ligands and the TLR4 ligand LPS for their capacity to induce IL-10 production in human B cells (see Fig. 2A-C, Note 8).

1. For flow cytometric detection of IL-10, cells are washed once with complete RPMI and restimulated with PMA (100 ng/ml) and ionomycin (1 µg/ml) in RPMI at 37°C, 5% CO_2 and humidified atmosphere. After two hours, Brefeldin A is added at a concentration of 10 µg/ml. In total, the cells are cultured under these conditions for six hours.
2. *Optional:* After restimulation with PIB, cells are harvested, washed with PBS and stained with Aqua dead cell staining kit (diluted 1:400 in PBS) for 15 min at room temperature.
3. The cells are fixed with 1.9% paraformaldehyde for 15 min at room temperature and stored at 4°C or -20 °C.
4. For intracellular staining, the cells are first washed once with permeabilization buffer, and then apply 50 µl/well antibody mix and incubated for 30 minutes at 4°C (see details Section 2.4).
5. After washing once with permeabilization buffer, cells are resuspended in FACS buffer and measured on a FACS Canto or similar instrument (see Note 8, Fig. 2).

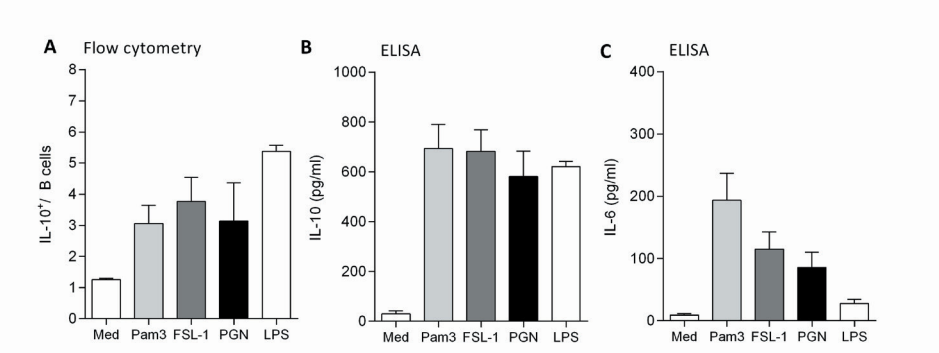


Figure 3. IL-10 and IL-6 expression of murine B cells in response to TLR stimulation. CD19$^+$ sorted splenic B cells were stimulated with the TLR ligands LPS (100 ng/ml), Pam3-Cys, FSL-1, and PGN (each 10 µg/ml), or cultured in medium as a negative control. (A) The frequency of IL-10 positive B cells was determined after 3 days stimulation followed by 6 h culture with PMA/ionomycin (BrefeldinA added in the last 4 h). Secreted IL-10 (B) and IL-6 (C) was measured by ELISA after 5 days stimulation with TLR ligands. Mean ± SEM of 3 (A) or 4 (B, C) independent experiments is given.

3.3.2 Quantification of cytokine expression by ELISA

As TLR ligation not only induces IL-10 but also other pro-inflammatory cytokines (e.g. IL-6, TNF-α), cytokine expression of various cytokines (so not only IL-10) should be taken into account to determine whether certain TLR ligands truly induce an anti-inflammatory response or not. For optimal Breg activity, a TLR ligand should display a strong IL-10 and a low IL-6 inducing capacity. For example, the TLR2 ligand PGN induced more human B cell IL-10 secretion in the supernatant compared to the other TLR2 ligands, but also the highest IL-6 production (Fig. 2B, D and Note 9). In contrast, for mouse B cells, while several TLR2 ligands induced similar levels of secreted IL-10, IL-6 levels were lowest with PGN, suggesting that in this case PGN is a more potent Breg cell inducing TLR2 ligand in mouse cells *in vitro* (Fig. 3). Of note, the TLR4 ligand LPS showed by far the highest IL-10/IL-6 ratio in murine B cells (Fig. 3 B,C).

1. Supernatants from TLR-stimulated cells are collected and stored at -20°C (see Note 9).
2. *Optional:* After TLR-priming, the cells are cultured 1:1 with CD40L expressing cell line (J558 cells, irradiated with 2600 RAD) to mimicking B and T cell interaction for an additional 24 hours. Next, the supernatant is stored at -20 °C (see Fig. 2C, Note 8).
3. *Optional:* After TLR-priming, the cells are restimulation with PMA/ionomycin/ BrefA (see section 3.3.1) and supernatant containing cytokines produced in two hours after PMA/ionomycin is stored at -20°C.
4. ELISA coating antibodies, detection antibodies and standards are used in concentrations as recommended by the manufacturer.

3.4 Detection of Tr1/FoxP3 T cell by flow cytometry after co-culture

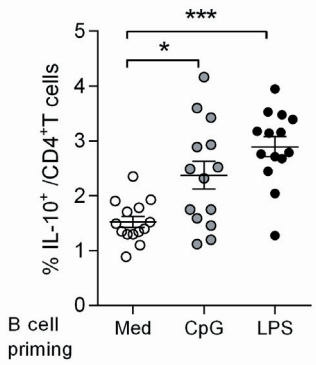


Figure 4. IL-10$^+$ T cells in co-cultures of TLR-primed B cells and T cells. B cells were stimulated for 48 h by medium, CpG (5 μg/ml) or LPS (100 ng/ml), followed by a 1:1 co-culture with autologous memory CD4$^+$ T cells for 6 days. Next, the cells were restimulated with PMA/ionomycin (BrefeldinA added in the last 4 h) for 6 h. Percentage of IL-10 producing CD4$^+$ T cells was determined by flow cytometry.

1. Even when B cells secrete elevated levels of IL-10, it is essential to elucidate whether the B cells from the test conditions really have obtained suppressive properties to confirm their regulatory B cell status (see Note 11). Most studies compared the suppressive activity of B cells from autoimmune or allergic patients with healthy controls using different culture systems *ex vivo*. In this context, we have set-up an *in vitro* co-culture assay with human B cells from healthy donors which were first primed with TLR ligands, followed by culture with autologous CD4$^+$ memory T cells to investigate their acquired function capacities (24) as described below. Figure 4 shows an example of LPS- and CpG-primed B cells both induce a clear increase in IL-10-producing T cells compared to medium-primed B cells, suggesting they have acquired an increased regulatory capacity in B cells as a consequence their TLR ligation (see Note 11, Fig. 4).
2. 1 x 10^5 human B cells are stimulated via TLR 4 (ultra-pure LPS) or TLR9 (CpG) for two days (total volume 200 μl/well) (see Section 3.2).
3. Isolated memory CD4$^+$ T cells (see Section 3.1.2) are stored at 4°C for two days.
4. After two days, supernatant is stored, and the cells are washed, and co-cultured with autologous memory CD4$^+$ T cells (ratio 1:1) in RPMI supplemented with 10% FCS for an additional six days.
5. The cells are restimulated by PMA/ionomycin and BrefA (as indicated above) and fixated with FoxP3 fixation/permeabilization buffer (eBioscience). The cells were stained for flow cytometry as indicated in Section 2.4.

4. Notes

1. Isolation of B cells using e.g Miltenyi Biotec beads reaches approximately 95-98% purity. Check for each isolation the contamination of other cells, especially dendritic cells and monocytes, as they being very responsive to TLR ligand stimulation. Depletion of other cells, prior to $CD19^+$ B cell isolation, should reduce their putative contamination.
2. The concentration of anti-mouse CD19 magnetic beads used for isolation can be reduced after empirical testing of the sorting efficiency. For C57BL/6 splenic B cells, 7 µl instead 10 µl beads per 10^7 cells can be used without reduction in cell purity.
3. PBMC and B cell isolation should be performed preferably directly after blood draw. In addition, it is recommended to use fresh B cells and not live cryopreserved PBMC or B cells, because the B cell cytokine responses are diminished after freezing and thawing.
4. Before applying these assays, the number of cells in the culture and the duration of TLR ligation should be considered. We have established that higher cell numbers of human B cells are needed to evaluate secreted cytokine levels in response to weak TLR ligands, such as LPS. A minimum of 3.0 x 10^6 B cells/ml (3.0 x 10^5/well) showed detectable levels of secreted IL-10 and IL-6 (approx. 30 pg/ml IL-10 and 612 pg/ml IL-6 above medium) in response to 100 ng/ml LPS and therefore this cell number was used in further experiments (data not shown).
5. Kinetic experiments showed that cytokine production by human B cells reached its maximum after two days of TLR exposure and longer incubations even seemed to reduce the concentration in the supernatant, which may be explained by the assumption that cytokines in the supernatant will be consumed; for example, IL-10 acts as an autocrine factor promoting expansion of IL-10-producing B cells or differentiation of B cells into plasma cells (45;46). Although in murine B cells the TLR4 expression is more abundant compared to human B cells, a minimum of 3.0 x 10^6 cells/ml stimulation of five days resulted in the most optimal cytokine detection (data not shown). Therefore, it is recommended to apply the cell numbers and time points described above to allow detecting of cytokines in response to 'weak' TLR ligands, like LPS. In case the strength of the stimulus of interest or combinations of stimuli is unknown, additional kinetic or dose range studies are recommended.
6. For flow cytometric analysis of B cell IL-10 expression, a two (human) to three (mouse) days stimulation is recommended instead of five days, because of a better cell viability.
7. Stimulation of cells should be performed at least in duplicates in order to increase the accuracy of the ELISA results.
8. Flow cytometry and ELISA may not be fully comparable because for intracellular cytokine visualization by flow cytometry, an additional restimulation, such as

PMA/ ionomycin/ BrefeldinA (PIB) is needed (see section 3.3.1). An example was represented in Figure 2, showing that applying intracellular cytokine staining, both TLR1/2 ligand Pam3CysK and TLR2/6 ligand FSL-1 induced more B cell IL-10 compared to peptidoglycan (PGN, TLR2/CD36) in human B cells (Fig. 2A), while surprisingly, PGN induced the highest IL-10 secretion as measured by ELISA (Fig. 2B). To sort out whether the second stimulation (PMA/ionomycin) changes the B cell response, TLR2 primed B cells were stimulated either for two hours with PMA/ionomycin (regular protocol for intracellular cytokine staining) or for 24 hours with a CD40L expressing cell line J558 to mimic B and T cell interaction in a more physiological manner (Fig. 2C). Both after PMA/ionomycin stimulation and after CD40 ligation, a higher IL-10 secretion was detected in FSL-1- and Pam3CysK- primed B cells in the supernatant, but not for the PGN-primed B cells. This indicates that in this example the second (putative T cell mediated) stimulation acts as an amplifying signal for B cell IL-10 production for certain TLR2 ligands (but not for all), and which is mimicked by PIB restimulation. Therefore, IL-10 production as marker for Breg cell induction by different TLR ligands should be confirmed both by flow cytometry and ELISA to be able to understand the role of certain TLR ligands for B cell activity under innate conditions and after interaction with T cells. Of note, the intracellular IL-10 levels measured by flow cytometry will be more representative for their behavior in the interaction with T cells.

9. It is important to investigate both pro- and anti-inflammatory cytokine responses when studying the role of TLR ligands as promoting adjuvant for Breg cells, and to consider species-related differences when extrapolation findings from disease models to human conditions. Thus, the Breg inducing capacity can be diverging for the same TLR ligand with different species (e.g. here: PGN for mouse and human B cells), as well as for different ligands targeting the same TLR (here: TLR2, see Fig. 2 and 3).
10. To correct for background staining of other fluorochromes in the staining mix, using FMOs (fluorescence minus one) for IL-10 (and other cytokines) is recommended. At the moment, FMOs are regarded as the best control in multicolor experiments and can also help to rule out problems in compensation or aspecific binding of the antibodies. For reference on this policy, see also Roederer *et al.* 2001 Cytometry (47), Herzenberg *et al.* 2006 Nature (48), Maecker *et al.* 2006 Cytometry (49) and TreeStar Daily Dongle 2011.
11. Next to the identification of IL-10 and other cytokine production, the functionality of the IL-10-producing B cells needs to be investigated before the term 'Breg cell' can be applied. This can be performed using memory T cells (Fig. 4) but also using different cell types as described by other groups (20;21;33) e.g. for their capacity to reduce cytokines or cell proliferation.

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