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Cellular Immunology

Stimulation with FITC-labeled antigens confers B cells with regulatory properties

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Response to Reviewers:	

Dear Editor

Please find here the revised version of our article entitled “Stimulation with FITC-labeled antigens confers B cells with regulatory properties” by Planchais et al.

We believe that we have now addressed all the reviewer’s concerns. In particular we have corrected and added references, removed “data not shown” from the text, enriched the discussion by acknowledging a potential biasing effect by a putative contamination of OVA-FITC by LPS, removed mention to B10 cells, added a supplementary Figure to substantiate FACS data shown on Figure 4B and clearly indicated that the EAE experiment was performed twice.

We hope that our article is now acceptable for publication and remain open to any question or suggestion for improvement.

Lastly, we would like to apologize for the time taken to reply to the reviewer’s queries. The work has been completed several years ago and the PhD student had problems to find time to recover old data files.

Thank you again for your support.

Kind regards

Sebastien Lacroix-Desmazes

Title: Stimulation with FITC-labeled antigens confers B cells with regulatory properties

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Abstract

B cells with regulatory properties (Bregs) were identified in human and in mice among different B-cell subsets. Their regulatory properties rely mainly on the production of anti-inflammatory cytokines, in particular IL10, IL-35 and TGF β , and were extensively studied in mouse models of autoimmune and inflammatory diseases. However, the exact nature of the stimulatory signals conferring regulatory properties to B cells is still not clear. We serendipitously observed that fluorescein isothiocyanate (FITC) binds to a significant proportion of naïve mouse B cells. Binding of FITC to the B-cell surface implicated at least in part the B-cell receptor. It triggered IL-10 production and allowed the endocytosis of FITC-coupled antigens followed by their presentation to CD4⁺ T cells. In particular, B cells incubated with FITC-OVA polarized OTII T cells towards a Tr1/Th2 phenotype *in vitro*. Further, the adoptive transfer of B cells incubated with FITC-labeled myelin oligodendrocyte glycoprotein peptide protected mice from experimental autoimmune encephalomyelitis, a T-cell-dependent autoimmune model. Together, the data show that FITC-stimulated B cells polarize immune responses towards Tr1/Th2 and acquire immuno-modulatory properties.

Keywords. Immuno-modulatory B cells, IL-10, experimental autoimmune encephalitis

Abbreviations. Bregs, regulatory B cells; EAE, experimental autoimmune encephalitis;

FITC, fluorescein isothiocyanate; BCR, B-cell receptor

Introduction

B cells with regulatory properties (Bregs) have been extensively studied over the past decade due to their therapeutic potential in the fields of autoimmunity and transplantation. Apart from therapeutic usage, Bregs may also exert pathogenic properties in human alloimmune and infectious diseases (1,2). In mice, regulatory properties have been attributed to phenotypically diverse B-cell sub-populations such as B1, marginal zone (MZ) and transitional 2 (T2) B cells (3–5). Various studies identified interleukin-10 (IL-10) as the critical effector of the regulatory potential of B cells. Indeed, B cells from IL-10-deficient mice are unable to protect against disease development. Apart from IL-10, Bregs express other immune-regulatory cytokines including IL-35 and transforming growth factor β (TGF- β) (6–8). Bregs can be induced following stimulation of CD40 (4), Toll-like receptors (TLRs) (3), CD80, CD86 or the B-cell receptor (BCR) (9). In several murine models, the mechanism of the Breg-dependent tolerance appears to be mainly antigen-specific (10–12). Yet, because of the lack of Bregs-specific surface markers and due to the scarcity of antigen-specific B cells in the circulation, the use of Bregs as effective agents for cell therapy remains to be made demonstrated. We have serendipitously observed that fluorescein isothiocyanate (FITC) binds to the BCR of a significant proportion of naive mouse B cells, and that the binding of FITC coupled to model antigens to the BCR triggers the endocytosis of the FITC-coupled antigen, and fosters the production of IL-10 by a substantial percentage of naive B cells. The B cells in turn influence the polarization of CD4 Th0 cells toward Th2/Tr1 cells. Here, we validated the immunoregulatory properties of FITC-stimulated B cells *in vitro* as well as *in vivo* using the model of experimental autoimmune encephalomyelitis (EAE).

Materials and methods

Animals, antigens and cell clones

Eight to 9-week-old BALB/c mice (males), C57BL/6 mice (females and males) and OTII mice (males) were obtained from Charles River (France). BCR-deficient V_H-LMP2A mice (males) (13) on BALB/c background, were provided by Dr Christophe Sirac (UMR CNRS 6101, Limoges, France). Inbred C57BL6/J-exon 16 FIII-deficient male mice were grown bred at the pathogen-free animal facility of Cordeliers Research Center, Paris. All animal studies were performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (UPMC Paris) at the pathogen-free animal facility of Cordeliers Research Center, Paris. FITC-labeled and biotinylated MOG₃₅₋₅₅ peptides were purchased from PolyPeptide Laboratories (Strasbourg, France) and FITC-labeled ovalbumin (OVA) from Invitrogen (OVA-FITC, life technologies, Saint Aubin, France, contains 4 moles of FITC for 1 mole of OVA).

Ovalbumin (Sigma-Aldrich, Saint-Quentin Fallavier, France) was labeled with biotin using EZ-Link* NHS-LC-Biotin (Thermo scientific, Illkirch, France); the excess of free biotin was removed by dialysis against Phosphate-buffered saline (PBS). Cells were cultured in RPMI medium (Lonza, Levallois-Perret, France) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-ME, 0.1 mM non-essential amino acids and 10% of heat inactivated fetal bovine serum. DO54.8 is an OVA₃₂₃₋₃₃₉-specific mouse CD4⁺ T hybridoma restricted to I-A^d (C57BL/6).

Purification of B and T cells

B cells were purified from the spleen of BALB/c, V_H-LMP2A or C57BL/6 mice using either the “Pan B cell isolation kit” or CD19 MicroBeads (MACS, MiltenyiBiotec, Paris, France). Briefly, splenocytes were isolated after mechanical dilaceration of spleens of naïve mice and

splenocytes were incubated 90 s in ACK lysis buffer (life technologiesTM, Invitrogen) at room temperature. Cells were then passed through 70 μ m nylon membrane filters and suspended in PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA, Sigma-Aldrich) as recommended by the manufacturer. CD4⁺ T cells were purified from splenocytes of OTII and wild-type C57BL/6 mice using Dynabeads[®] Mouse CD4 (life technologyTM, Invitrogen). Total cells were also purified from the blood and lymph nodes of mice. In brief, blood cells were collected upon perfusion of the mice with 40 ml of 0.2 mM EDTA in PBS through the left ventricle. Draining lymph nodes were collected and mechanically dilacerated. Single cell suspensions were prepared as described above.

Binding and endocytosis tests

Binding of antigens to cell surface was evaluated using total or negatively selected B cells from splenocytes of naïve mice. Cells were incubated 1 hr on ice alone or in the presence of antigens. After incubation, cells were washed and stained with a PE-labeled anti-mouse CD19 antibody (1D3; BD biosciences, Le Pont de Claix, France) for 25 min at 4°C. Cells were then incubated for 25 min with APC-labeled annexin V (Invitrogen), washed and incubated with propidium iodide (Invitrogen) prior to FACS analysis. Binding of antigens was evaluated by flow cytometry (BD LSRII) either by direct fluorescence of FITC or using Pacific Blue-coupled streptavidin (Molecular Probes, Invitrogen) on the viable cell population (i.e., negative for annexin V and propidium iodide). Endocytosis of antigens was performed using negatively selected B cells purified as described above. B cells were incubated alone or in the presence of antigens for 1 hr at 4°C or 37°C. After incubation, B cells were washed, stained with a PE-labeled anti-mouse CD19 antibody (1D3; BD biosciences) for 25 min at 4°C, and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences, Le Pont de Claix, France). When indicated, the FITC and Pacific blue fluorescences were normalized using FITC-labeled and Pacific Blue-

labeled anti-CD19 IgG (clone 1D3). Endocytosis of antigens was evaluated by subtracting the normalized mean of fluorescence intensity (MFI) obtained at 37°C from that obtained at 4°C. Cells were acquired using a BD LSR II and analyzed with FACS Diva or FlowJo software (BD Biosciences).

Analysis of T-cell activation and polarization

The DO54.8 T-cell hybridoma ($2.5 \cdot 10^5$ cells) was incubated alone or with negatively selected B cells ($2.5 \cdot 10^5$ cells) from BALB/c mice in complete medium with or without FITC-labeled or biotinylated-OVA (10-200 µg/ml) for 2 days at 37°C. The supernatant was collected and the production of IL-2 by DO54.8 was quantified using a mouse IL-2 ELISA Set (BD OptEIA10™, BD bioscience). For T cell polarization, purified CD4⁺ T cells from OTII mice were labeled with CellTrace™ violet (CTV, life technology™, Invitrogen). Briefly, purified CD4⁺ T cells (10^6 cells/ml) were incubated with CTV (3 µM) for 20 min at 37°C in complete medium and washed to remove the excess of free dye. B cells (10^5 cells) from C57BL/6 mice were co-incubated with purified CD4⁺ T cells (10^5 cells) with or without OVA-FITC (5-20 µg/ml) for 6 days at 37°C. When indicated, B and T cells were incubated with a neutralizing anti-IL-10 antibody (JES3-19F, Biolegend, San Diego) or with a neutralizing anti-IL-10 receptor antibody (clone 1B1.2, a kind gift from Dr Sylvaine You (Institut Necker-Enfants Malades, Paris, France)). For intracellular cytokine staining, cells were stimulated *in vitro* with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich) in the presence of Ionomycin (1 µg/ml, eBioscience, Paris, France) and Monensin (GolgiStop®, BD biosciences) for 6 hr at 37°C. T-cell polarization was evaluated using PE-Cy5-labeled anti-CD4 antibody (H129.19, BD biosciences), PE-labeled anti-IL-10 antibody (JES5-16E3, BD biosciences), APC-labeled anti-IFN γ antibody (XMG1.2, BD biosciences), Alexa 700-labeled anti-FoxP3 antibody (FJK-16s, eBioscience) and the FoxP3 staining buffer set (ebioscience) by flow cytometry.

Analysis of B-cell activation

Negatively selected B cells (10^6 /ml) from C57BL/6 mice were incubated alone, in the presence of $F(ab')_2$ fragments of an anti-mouse IgM antibody (10 μ g/ml, Jackson ImmunoResearch), of OVA-FITC (200 μ g/ml) or of OVA-biotin for 2 days at 37°C. B-cell activation was evaluated using Pacific blue-conjugated anti-CD19 (1D3), PE-conjugated anti-CD80 (16-10A1, BD biosciences), APC-conjugated anti-CD83 (Michel-19, BD biosciences), PE-conjugated anti-I-Ab/I-Eb(2G9, BD biosciences) and biotinylated anti-CD86 (GL1, BD biosciences) associated with the Alexa-fluor® 700 labeled streptavidin (Molecular probes®, Invitrogen). Briefly, 10^6 B cells on day 2 were washed and suspended in 100 μ l of ice-cold PBS containing 0.5% BSA and secondary antibodies (2 μ g/ml final concentration of each) for 25 min on ice. B cells were then washed and suspended in 300 μ l of PBS/0.5% BSA supplemented with propidium iodide (5 ng/ml). The level of expression of surface was evaluated by flow cytometry with FACS Diva software.

Analysis of B-cell subpopulations.

Total splenocytes were isolated from naïve mice as previously described. Cells were surface stained with a combination of PE-labeled anti-CD21/CD35 antibody (4E3, ebioscience), PE-Cy7-labeled anti-CD23 antibody (B3B4, ebioscience), APC-labeled anti-CD19 antibody (1D3), and PE-labeled anti-CD24 antibody (M1/69, BD Pharmingen) associated with the Alexa-fluor® 700-labeled streptavidin (Molecular probes®, Invitrogen). The marginal zone B cells (MZ) were referred as $CD24^- CD21^{++} CD23^{low}$, the follicular B cells (FO) as $CD24^- CD21^{low} CD23^+$, the transitional 1 B cells (T1) as $CD24^+ CD21^- CD23^-$ and the transitional 2 B cells (T2) as $CD24^+ CD21^+ CD23^+$.

Cytokine production by B cells

Purified B cells were obtained as described above. B cells (10^6 cells/ml) were incubated alone

or in the presence of antigens (200 µg/ml) for 2 days at 37°C. Supernatant was stored at -80°C and B cells were stimulated *in vitro* for intracellular cytokine staining as previously described. Intracellular staining was evaluated using APC-labeled anti-CD19 antibody (clone 1D3), PE-labeled anti-IL-10 antibody (JES5-16E3, BD biosciences) and the Cytotfix/Cytoperm kit (BD Biosciences) by flow cytometry. Quantification of cytokines secreted by B cells in the supernatant was assessed using the BD *CBA Mouse Inflammation Kit* (BD biosciences).

Induction of EAE

Female C57BL/6 mice were immunized with 100 µg MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, v/v 1:1) containing 400 µg of *Mycobacterium tuberculosis* (Difco Laboratories, L'Arbresle, France). A final volume of 200 µl was injected subcutaneously at 2 sites over the flanks. In addition, 150 ng of Pertussis toxin (List Biologic Laboratories, Meudon, France) was given intravenously on the same day and 2 days later. Clinical signs of EAE were assessed daily by the following scoring system: 0, no sign; 1, hindlimb weakness; 2, hindlimb weakness and tail paralysis; 3, hindlimb and tail paralysis; 4, hindlimb and tail paralysis and forelimb weakness; 5, moribund.

B-cell transfer experiments

On day five and day one before EAE induction, as well as on day five after induction of the disease, mice received intravenously B cells (10⁶ cells) from naïve animals incubated alone or in the presence of FITC-labeled or biotinylated antigens for 3 hr at 37°C in HL-1 medium (Lonza).

Statistics

All data are shown as mean ± SEM (n between 3 and 10, as indicated). Statistical significances

were assessed using the double-sided non-parametric Mann Whitney U test. We used the two-way ANOVA with Bonferroni's post-hoc t test for comparing EAE scores (* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$).

Results

B cells from naïve mice bind FITC-labeled protein in a BCR-dependent manner

We first investigated the capacity of splenic B cells from naïve wild-type mice to bind FITC. To this end, we used FITC coupled to OVA as a carrier protein. FITC-labeled OVA (200 µg/ml) was incubated for 1 hr at 4°C with splenocytes purified from naïve C57Bl/6 mice. Binding was analyzed by flow cytometry. Since binding of FITC at the surface of B lymphocytes has been documented previously (14), the usage of FITC-labelled isotype IgG was not conceivable to set thresholds; the threshold of FITC positivity was therefore based on the CD19-negative population. Under such conditions, OVA-FITC-positive B cells represented $9.9\pm 1.3\%$ (mean \pm SEM) of the total splenocytes (Figure 1A) and $16.4\pm 2.26\%$ of the CD19⁺ cells. Accordingly, the mean fluorescence intensities of FITC among the CD19⁺ and CD19⁻ populations were 263 ± 21.7 and 84 ± 10 , respectively. Binding of OVA-FITC to the surface of purified CD19⁺ cells increased in a dose-dependent manner and reached a plateau of >25% positive cells at 800 µg/ml (Figure 1B). Interestingly, biotinylated OVA (~~as well as native OVA, data not shown~~) failed to bind the surface of CD19⁺ cells, indicating that binding is mediated by the FITC moiety rather than OVA. Importantly, 15 to 25% of purified CD19⁺ cells bound FITC-coupled MOG₃₅₋₅₅ peptide, as well as FITC-coupled factor IX and FITC-coupled factor VIII (Figure 1C, all at 4 µM), while no binding was observed with the biotinylated forms of the molecules. This indicates that the nature of the antigens does not influence the FITC-mediated antigen binding on the B-cell surface. The binding of FITC to B cells was mediated at least in part by the BCR as shown by the absence of OVA-FITC binding to B cells isolated from V_H-LMP2A BCR-deficient (BCR^{-/-}) BALB/c mice (13), when compared to B cells from naïve wild-type BALB/c mice ($20.3\pm 1.7\%$) (Figure 1D).

We then investigated whether the binding of OVA-FITC by CD19⁺ cells is related to the natural broad reactivity of some B-cell sub-populations. Different percentages of inguinal lymph nodes

and peritoneal B cells bound OVA-FITC ($21.1\pm 2.2\%$ and $60.8\pm 4.7\%$, respectively, Figure 2A). Similarly, the different splenic B-cell subsets, T1, FO, T2 and MZ B cells, bound OVA-FITC to different extents (16.2 ± 1.9 , 16.8 ± 1.8 , 31.5 ± 6.4 and $29\pm 4.5\%$, respectively, Figures 2C and 2D). Interestingly, the levels of expression of surface IgM by the different splenic B cell subsets, correlated positively with the intensity of binding of OVA-FITC (Figures 2E and 2F). All together, these results suggest that the binding of the FITC moiety by the B cells is not restricted to a specific subset of B cells but is strongly related to the level of immunoglobulin expression on the B cell surface.

FITC-labeled OVA is endocytosed and presented by naive splenic B cells

We then explored the consequences of FITC binding to the BCR of naive B cells on their antigen presentation capacity. Purified naive B cells endocytosed FITC-labeled OVA in a dose-dependent manner, but failed to endocytose biotin-labeled OVA (Figure 3A). Co-incubation of purified splenic B cells from BALB/c mice with the OVA₃₂₃₋₃₃₉-specific CD4⁺ DO54.8 T cells hybridoma in the presence of OVA-FITC, lead to a dose-dependent activation of DO54.8 T cells (Figure 3B). T cells incubated with B cells alone, with biotinylated-OVA (Figure 3B) or ~~unlabeled OVA~~, or incubated with OVA-FITC in the absence of B cells, failed to secrete IL-2 ~~(data not shown)~~.

Binding of FITC-labeled OVA to B cells induces IL-10 production in vitro

We then evaluated and compared the cytokines secreted by purified splenic B cells incubated either with a BCR cross linker (anti-IgM F(ab')₂ fragment) or with OVA-FITC *in vitro*. When stimulated for 24 hr with anti-IgM F(ab')₂ fragments, purified splenic B cells secreted high amounts of TNF α and MCP-1 as compared to control B cells (Figure 4A). In contrast, B cells stimulated with OVA-FITC (200 μ g/ml) secreted high amounts of IL-10 as compared to cells

incubated alone, with anti-IgM F(ab')₂ fragments or with OVA-biotin. Because stimulation with OVA-FITC triggered the production of IL-10 by B cells, we investigated whether the binding of OVA-FITC is restricted to previously described population of IL-10 secreting regulatory B cells **expressing high level of CD1d and CD5 markers** (5). OVA-FITC-binding B cells belonged to both the **CD19⁺CD1d^{hi}CD5⁺** and **CD19⁺CD1d^{low}CD5⁻** cell-subpopulations (Supplementary Figure 1). OVA-FITC-binding **CD19⁺CD1d^{hi}CD5⁺** cells however accounted for 0.56±0.29% of the total splenic OVA-FITC-positive B cells.

We then compared the activation markers expressed by B cells stimulated by anti-IgM F(ab')₂ fragments or OVA-FITC. Naive B cells incubated with anti-IgM F(ab')₂ fragments underwent strong activation which was not the case when B cells were incubated with OVA-FITC (Figure **4B** and **Supplementary Figure 2**), as evidenced by the percentage of cells expressing CD86 (75.8±9.3 versus 24.1±3.9%, respectively, p=0.003), CD83 (66.5±3.9 versus 2.7±0.9%, respectively, p=0.0004) and CD19^{high} (18.8±2.2 versus 9.3±0.9%, respectively, p=0.02). Likewise, the MFI of HLA class II was drastically increased upon B cell stimulation with anti-IgM F(ab')₂ fragments (44.7×10³±8.5×10³) as compared to OVA-FITC (11×10³±3.4×10³, p=0.01). The incubation of B cells alone or in presence of OVA-biotin failed to induce B-cell activation (Figure **4B**).

Presentation of FITC-labeled OVA by naïve splenic B cells polarizes CD4⁺ T cells toward Th2/Tr1 cells

We explored the consequences of BCR-mediated FITC binding to the BCR of naive B cells on their antigen presenting capacity to polarize the CD4⁺ T cell response. The proliferation and polarization of primary OVA₃₂₃₋₃₃₉-specific CD4⁺ T cells following incubation with negatively selected OVA-FITC-stimulated B cells was assessed using naive CD4⁺ splenic T cells purified from OTII mice. Incubation of CD4⁺ OTII T cells with ConA, used as a positive control,

induced the proliferation of $38.2 \pm 1.7\%$ of the total $CD4^+$ T cells (Figure 3C, upper panels). The presence of OVA-FITC increased in a dose-dependent manner the percentage of proliferating T cells (up to $27.8 \pm 0.98\%$). In the presence of ConA, $0.6 \pm 0.1\%$ and $2.3 \pm 0.2\%$ of the $CD4^+$ OTII T cells were positive for intracellular $IFN\gamma$ and IL-10, respectively (Figure 3C, bottom panels), while most of the OTII T cells incubated alone were negative for $IFN\gamma$ and IL-10. The co-culture of OTII $CD4^+$ T cells and C57BL/6 B cells in the presence of OVA-FITC lead to a dose-dependent generation of $IL-10^+ CD4^+$ T cells, and a marginal increase in percentages of $IFN\gamma^+ CD4^+$ T cells. Importantly, there was no generation of FoxP3-positive $CD4^+$ T cells (~~data not shown~~). In the presence of ConA, both dividing (CTV^{low}) and non-dividing (CTV^{high}) OTII T cells were negative for IL-10 expression (Figure 3D). In the presence of C57BL/6 B cells and OVA-FITC, the percentages of $IL-10^+ CD4^+$ OTII T cells increased in a dose-dependent manner up to $36.8 \pm 2.8\%$ and $16.1 \pm 1.4\%$ for dividing and non-dividing OTII T cells, respectively (Figure 3D).

Adoptive transfer of MOG₃₅₋₅₅-FITC-loaded naive B cells protects mice from EAE

We then investigated the protective potential of adoptively transferred naive splenic B cells pre-incubated with FITC-labeled MOG₃₅₋₅₅ peptide in EAE, an experimental model of multiple sclerosis. Recipient mice received purified splenic B cells (10^6 cells) from naive mice pre-incubated alone, with biotin-MOG₃₅₋₅₅, with FITC-MOG₃₅₋₅₅, or with OVA-FITC. Following induction of EAE, mice injected with untouched purified B cells or with B cells incubated with MOG-biotin developed the first clinical signs at day 10-11, with a peak of the disease at day 17 and with a mean clinical score of 2.85 ± 0.38 and 2.89 ± 0.27 , respectively (Figure 5A). Mice injected with B cells incubated with OVA-FITC exhibited a delayed onset of the disease (day 19) but the severity remained comparable to that of the control group with a mean clinical score of 2.85 ± 0.38 (Figure 5A). The transfer of B cells pre-incubated with MOG₃₅₋₅₅-FITC not only

delayed the onset of the disease (day 14), but also significantly decreased the severity of the disease (mean of clinical score of 1.22 ± 0.31 at day 18). At day 18, representative mice that received B cells pre-incubated with FITC- or with biotin-labeled MOG₃₅₋₅₅ were sacrificed and cells from the spleen, blood and draining lymph nodes (DLN) were isolated. Percentages ~~as well as absolute numbers~~ of IL-10-positive CD4⁺ T cells (Figure 5B ~~and data not shown, respectively~~) increased significantly in the blood, DLN and spleen of mice adoptively transferred with naive B cells pre-incubated with MOG₃₅₋₅₅-FITC (Figure 5B). Analysis of the IL-10⁺CD4⁺ T-cell populations for the expression of FoxP3 showed no statistical difference in any of the tested organs between the 2 groups of mice, in agreement with *in vitro* data. The percentage of IFN γ ⁺CD4⁺ T cells was marginally although statistically higher (P=0.042) in the DLN of mice that received B cells pre-incubated with MOG₃₅₋₅₅-FITC, ~~but absolute numbers of IFN γ ⁺CD4⁺ T cells were not statistically different between the 2 groups of mice (data not shown)~~.

Discussion

Our results indicate that stimulation of naïve B cells by protein-coupled FITC polarizes B cells towards an immunoregulatory phenotype. Notably, the adoptive transfer of naïve B cells stimulated by MOG-FITC reduces disease severity in EAE. Several pieces of evidence suggest that FITC is a promiscuous BCR ligand. Incubation of OVA-FITC, used as a model antigen, with total splenic B cells from naïve mice resulted in a 3-fold increase in mean fluorescent intensity of the B-cell population as compared to the CD19-negative population. Besides, FITC-labeled antigens failed to bind to the surface of B lymphocytes from BCR^{-/-} V_H-LMP2A mice, suggesting that the binding to B cells is mediated by the BCR complex. Several approaches have been described to generate Bregs, wherein the involvement of the BCR is only indirectly suggested. In the arthritis model of DBA/1 mice immunized with collagen, IL-10⁺ B cells were generated *ex vivo* only provided that the B cells originate from immunized animals and are stimulated with an anti-CD40 antibody in the presence of the antigen (4). Moreover, in the case CD1d^{high}CD5⁺ B cells (15), stimulation with LPS, a recognized BCR ligand (16), was able to induce ~~B10 cells~~ IL-10 production by B cells from both wild-type and MyD88-deficient mice. Of note, BCR diversity (i.e., polyclonality) was demonstrated to be required for B cells to produce IL-10 upon LPS stimulation (15), also inferring that not all B cells are sensitive to IL-10 induction, and thus indirectly suggesting the implication of BCR signaling as a pre-requisite for IL-10 production. In addition, impeding in the BCR-mediated calcium influx exclusively on B cells resulted in a reduced IL-10 production following BCR and anti-CD40 stimulation (17). More recently, direct evidences in a mouse allograft tolerance and during RSV infection in the human, further emphasized the importance of BCR stimulation in the acquisition of immune-regulatory properties by B cells (1,10).

B cells stimulated with OVA-coupled FITC acquired a phenotype characterized by low expression of activation markers, by the low production of TNF α and elevated production of

IL-10, and by antigen presentation to CD4⁺ T cells. Such a phenotype is reminiscent of the tolerogenic phenotype of immature dendritic cells (18,19). FITC is a small polyaromatic compound that has no known relevance in physiology. Even if the molecular mechanisms explaining the natural reactivity of B cells toward FITC remain unclear, the binding of circulating immunoglobulins and BCR to aromatic compounds and to fluorochromes has already been described (20–23). Incidentally, the capacity of FITC to bind to B lymphocytes from naive mice or from the peripheral blood of healthy donors has been described previously, without a demonstration of the implication of the BCR in the phenomenon (14,24).

The nature of the B-cell population(s) targeted by aromatic compounds, and in particular by FITC, remains unclear. Up to 15 to 20% of the total splenic B cells bound FITC, suggesting that the binding is not restricted to a single set of V_H and V_L genes. Besides, all B-cell subpopulations in the spleen, as well as B cells from the lymph nodes and from the peritoneum were capable of binding FITC, with a preference for peritoneal B1 cells, and splenic T2 and MZ B cells. Altogether, our data demonstrate that FITC-binding is not restricted to a subset of splenic B cells and its intensity strongly dependent on the density of BCR expressed on the surface of the cell. Because FITC binding to BCR confers the target B cells with immunoregulatory properties, our data indirectly suggest that Bregs (at least defined by the capacity to produce IL-10) do not belong to a distinct subset of B cells. Conversely, any B cell may polarize towards an IL-10 producing B cell provided the presence of appropriate signals. **OVA is widely used in several animal models may be contaminated by LPS, a potent IL-10 inducer in B cells (25). Importantly, BCR^{-/-} B cells from V_H-LMP2A mice failed to produce IL-10 following stimulation OVA-FITC. We cannot however rule out the possibility that both BCR and TLR4 stimulations are necessary for IL-10 production by B cells. OVA-FITC stimulation of B cells from MyD88/TRIF double knockout mice would help to understand the possible involvement**

of the TLR4 signaling in the production of IL-10 by splenic B cells from WT mice. Thus, † The complete nature of these signals remains to be deciphered.

The protective role of Bregs has been exploited experimentally in a large array of experimental models for Th1-mediated autoimmune diseases (4,5,9,15,26–29). In the human, neonatal nBregs downregulated Th1 cell responses against RSV in an IL-10-dependent manner (1). Conventional Bregs have been shown to convert effector T cells into IL-10-producing T cells through the provision of IL-10 (26,29). Here, the adoptive transfer of MOG₃₅₋₅₅-FITC “educated” splenic B cells from naïve mice, reduced the severity of EAE in recipient mice. It was associated with an increased percentage of CD4⁺IL-10⁺FoxP3⁻ T cells reminiscent of the phenotype of the Tr1 or Th2 T-cell subsets. Conversely, the transfer of B cells “educated” with OVA-coupled FITC, that do produce IL-10 *in vitro*, failed to efficiently protect mice from EAE, highlighting the antigen-dependency of the conferred protection and suggesting the recruitment of endogenous MOG₃₅₋₅₅-specific CD4⁺ T by MOG₃₅₋₅₅-FITC “educated” Bregs. These results are in line with seminal works in the Breg field describing the importance of antigen specificity for the regulatory efficacy of IL-10-producing B cells in autoimmune diseases, allograft resistance models and infectious diseases (1,4,10). Taken together, our results pave the way towards the *ex vivo* generation of antigen-specific immuno-modulatory B cells using FITC-coupled proteins. Induced antigen-specific Bregs should represent novel therapeutic tools for the treatment of Th1-mediated inflammatory diseases.

Acknowledgements

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Authors contributions

Planned the work: CP, AP, JB, SVK, JDD, SLD

Performed experiments: CP, JR, SD, SP

Analyzed the data: CP, JR, SP, AV, AP, JDD, SLD

Wrote the report: CP, SLD

The authors declare no conflict of interest

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Figures legends

Figure 1. Naïve B cells bind FITC in a BCR-dependent manner. Total cells (10^6 cells/ml) from the spleen (A) of naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 μ g/ml, OVA-FITC) on ice for 1 hr in serum free medium. (B and C) Purified B cells from naïve C57BL/6 mice were incubated with FITC-labeled or biotinylated-antigens (0 to 800 μ g/ml in B, or 200 μ g/ml in C) on ice for 1 hr in serum free medium. (D) Purified B cells from naïve BALB/c mice or naïve BCR^{-/-} mice (V_H-LMP2A mice) were incubated alone or in presence of OVA-FITC (200 μ g/ml) for 1h at 4°C. The graphs depict the percentage of viable antigen-positive cells evaluated either by the direct fluorescence of the FITC moiety or using Pacific Blue-labeled streptavidin by flow cytometry (cell viability was >85%). Percentage of positive cells from at least three independent experiments is presented. Error bars represent SEM.

Figure 2. FITC binds to different B-cell subsets. Total cells (10^6 cells/ml) from the spleen (Spl), the lymph nodes (LN) or the peritoneum (Perit) of naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 μ g/ml, OVA-FITC) on ice for 1 hr in serum free medium. (A). Splenic transitional 1 and 2 (T1 and T2), the marginal zone (MZ) and the follicular (FO) B cells were identified according to the level of expression of the surface markers CD19, CD21, CD23 and CD24 (B and C). The percentages of cells binding OVA-FITC and the intensities of OVA-FITC binding to each B-cell subset were evaluated by the direct fluorescence of the FITC moiety by flow cytometry (cell viability was >85%) (D). The intensity of BCR expression by each B-cell subset was also evaluated (E) and correlated with the intensity of OVA-FITC binding (F). Error bars represent SEM for three mice.

Figure 3. Natural FITC-binding naive B cells endocytose FITC-labeled OVA and induce IL-10-producing CD4⁺ T cells. (A) Purified splenic B cells (10⁶ cells/ml) from naive C57BL/6 mice were incubated with FITC-labeled (full squares) or biotinylated-OVA (empty circles) at 0 to 800 µg/ml, for 1 hr on ice or at 37°C in serum free medium. Endocytosis of OVA was evaluated either by direct fluorescence of FITC or using Pacific Blue-labeled streptavidin on fixed and permeabilized cells by flow cytometry. The graph depicts the difference of mean fluorescence intensity between 37°C and 4°C. The MFI was normalized with respect to FITC-labeled anti-CD19 antibody or Pacific Blue-labeled anti-CD19 antibody, used as positive controls. (B) Purified splenic B cells (2.10⁵ cells) from naïve BALB/c mice were co-incubated with the OVA₃₂₃₋₃₃₉-specific hybridoma (DO54.8, 2.10⁵ cells) alone, in the presence of FITC-labeled (full bars) or biotinylated-OVA (empty bars) at 10 to 200 µg/ml for 48 hr at 37°C in complete medium. The graph depicts the concentration of IL-2 secreted by DO54.8 in the supernatant evaluated by ELISA. The statistical significance of the observed differences between B cells incubated with OVA-FITC or OVA-biotin was determined by the double-sided non-parametric Mann-Whitney U test: ***p*<0.01 and ****p*<0.001. (C and D) Negatively selected splenic CD4⁺ T cells (1.5x10⁵ cells) from OTII C57BL/6 mice were stained with CTV and co-incubated with purified splenic B cells (1.5x10⁵ cells) from naive C57BL/6 mice alone or in the presence of OVA-FITC (5-20 µg/ml) for 6 days at 37°C in complete medium. As a positive control, CD4⁺ T cells were incubated with 2 µg/ml of Concanavalin A (ConA). Cells were then washed and stimulated for 6 hr at 37°C in the presence of phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 µg/ml) and Monensin. The proliferation (C, top panels) and polarization (C, bottom panels; D) of CTV-stained CD4⁺ T cells were evaluated on fixed permeabilized cells by flow cytometry. Values in quadrants depict the percentages of cells positive for the different markers: CTV, IFN-gamma and/or IL-10, gated on the CD4-positive

population. Data in all panels are representative of three independent experiments. Error bars represent SEM.

Figure 4. Stimulation of naïve B cells by OVA-FITC leads to the production of IL-10. (A) Negatively selected splenic B cells (10^6 cells/ml) from naïve C57BL/6 mice were incubated alone, with F(ab')₂ fragments of an anti-IgM antibody (10 µg/ml), with FITC-labeled or biotinylated-OVA (200 µg/ml) for 2 days at 37°C in complete medium. The graphs represent the concentrations of TNFα, IL-10 and MCP-1 secreted by the B cells in the supernatant. Data are representative of three independent experiments. (B) The activation status of negatively selected splenic B cells (10^6 cells/ml) from naïve C57BL/6 mice incubated for 2 days alone, with F(ab')₂ fragments of an anti-IgM antibody (10 µg/ml), with FITC-labeled or biotinylated-OVA (200 µg/ml) was assessed by the surface expression levels of CD80, CD86, CD83, CD19 and of MHC-II. Data are representative of two independent experiments. Error bars represent SEM. Statistical significance was assessed using ANOVA test: ns: not-significant; *: $p < 0.05$ and **: $p < 0.01$.

Figure 5. Adoptive transfer of MOG₃₅₋₅₅-FITC-loaded naïve B cells protects mice from EAE. (A) EAE was induced in 8 to 9 week-old female wild-type C57BL/6 mice by immunization with MOG in Freund's adjuvant and injection of Pertussis toxin. EAE mice were intravenously administered with negatively selected splenic B cells from naïve C57BL/6 mice (10^6 B cells/mouse) five days and one day before induction of EAE, as well as 5 days after induction of EAE. Mice (10 mice/group) received B cells that had been incubated alone (full circles), with OVA-FITC (200 µg/ml, empty squares), with biotinylated (4 µM, empty circles) or FITC-labeled MOG₃₅₋₅₅ (4 µM, full squares), for 3 hr at 37°C in serum free medium. The graph depicts the mean clinical scores over a period of 30 days. Error bars represent SEM (n=10 mice

per group). Statistical significance of differences in EAE scores between groups was analyzed by the two-way ANOVA with Bonferroni's post-hoc t test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (B) Mice that received MOG₃₅₋₅₅-biotin loaded-B cells (empty circles) or MOG₃₅₋₅₅-FITC loaded-B cells (full squares) were sacrificed at day 18 after induction of EAE. The graphs depict percentages of CD4⁺ T cells from blood, draining lymph nodes (DLN) and spleen. The studied T-cell populations include CD4⁺ T cells that are positive for IL-10 or for IFN γ , and CD4⁺IL-10⁺ T cells that are positive for FoxP3 expression. Horizontal lines depict mean values and SEM. Statistical significance was assessed using the double-sided non-parametric Mann Whitney U test: ns: not-significant; *: $p < 0.05$. Data are representative of 2 independent experiments.

Supplementary material

Supplementary Figure 1. Both $C19^+CD1d^{hi}CD5^+$ and $C19^+CD1d^{low}CD5^-$ cells may bind FITC-labeled OVA. Total splenocytes (10^6 cells/ml) from naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 μ g/ml, OVA-FITC) on ice for 1 hr in serum free medium. Percentages of OVA-FITC positive B cells were measured on splenic $C19^+CD1d^{hi}CD5^+$ and $C19^+CD1d^{low}CD5^-$ B cells, using the direct fluorescence of the FITC moiety of viable cells by flow cytometry (cell viability was >85%). Data represent means \pm SEM after measurements performed on 3 individuals. Statistical significances were assessed using the double-sided non-parametric Mann-Whitney U test: ** $p<0.01$; **** $p<0.0001$.

Supplementary Figure 2. Absence of activation of naïve B cells by OVA-FITC. The activation status of negatively selected splenic B cells (10^6 cells/ml) from naïve C57BL/6 mice incubated for 2 days alone, with F(ab')₂ fragments of an anti-IgM antibody (10 μ g/ml), with FITC-labeled or biotinylated-OVA (200 μ g/ml) was assessed by the surface expression levels of CD80, CD86, CD83, CD19 and of MHC-II. The graphs depict histograms of raw flow cytometry data for one representative experiments. Values indicated on histograms represent % of cells positive for CD19, CD80, CD83 and CD86. In the case of MHCII, mean fluorescent intensities (MFI) are reported.

Reviewer 1

COMMENTS:

In this manuscript, Planchais and colleagues report on the possibility of using FITC as a trojan horse to promote the BCR-mediated capture of conjugated antigens inside mouse B cells, which subsequently can present the later to T cells, while also acquiring the capacity to produce IL-10. The manuscript is well-written, and the data are well presented. The findings are well suited for publication in Cellular Immunology, and I therefore recommend publication of this interesting work after the minor points listed below have been addressed:

[We would like to thank the reviewer for the positive and constructive remarks](#)

References:

In the introduction, the authors should correct their referencing for accuracy.

In the sentence “Bregs express other immune-regulatory cytokines including IL-35...”, they should quote Shen et al. Nature 2014 v507 p366, which is the first article reporting the regulatory role of B cell-derived IL-35.

[This has been changed as requested by the reviewer](#)

In the sentence “Bregs can be induced following stimulation of CD40, Toll-like receptors... “. They should quote Lampropoulou et al. J. Immunol. 2008 v180 p4763 instead of ref 4 as this is the first article establishing the key role of intrinsic TLR signaling in B cells for their regulatory function.

[This has been changed as requested by the reviewer](#)

In the results section, the reference {Scott, 1976 #6} is not formatted.

[This has now been corrected](#)

In general, the data indicated as “not shown” should be systematically shown or the statement should be removed.

[Mention to not shown data have been removed from the text](#)

Unfortunately, the authors do not exclude the possibility that a small amount of LPS contained in the employed tools (OVA-FITC for instance) contributes to the observed phenotypes *in vitro*. It is known that commercial OVA usually contains residual LPS. The utilization of MyD88xTRIF dKO B cells would have helped to control for this possible issue. The authors should include a statement in their discussion to highlight this possibility.

[We agree with the reviewer and have now added a specific paragraph in the discussion to address this point](#)

The authors mention in page 12 “B10 cells” to refer to CD1dhiCD5+ B cells. However, B10 cells have remained ill-defined, and eventually have been equaled to “IL-10+ B cells”. To avoid confusion around this point and for accuracy, the authors should remove the mention of B10 cells throughout their manuscript, and refer instead to the precise phenotype they are looking at, based on the expression of CD1d and CD5.

We agree with the reviewer. Any mention to B10 cells has now been removed

The authors should show representative FACS plots for Figure 4.

We are now indicating representative FACS histograms for Figure 4B as a new supplementary Figure 2.

They should indicate how many times the EAE experiment shown in Figure 5 was performed, which is currently missing. Experiments that were only performed once should be removed from the manuscript if this applies.

We now indicate in the legend to Figure 5 that the data were performed twice.

Highlights

- Fluorescein isothiocyanate (FITC) binds to ~20% of splenic B cells from naïve mice
- Binding of antigen-coupled FITC to B cells induces IL-10 production and antigen endocytosis
- OVA-FITC loaded B cells polarizes OTII T cells towards a Tr1/Th2 phenotype
- MOG-FITC-loaded B cells protects mice from EAE upon adoptive transfer

Title: Stimulation with FITC-labeled antigens confers B cells with regulatory properties

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Abstract: 173 words; main text: 4024

Figures: 5

Supplementary Figures: 2

Abstract

B cells with regulatory properties (Bregs) were identified in human and in mice among different B-cell subsets. Their regulatory properties rely mainly on the production of anti-inflammatory cytokines, in particular IL10, IL-35 and TGF β , and were extensively studied in mouse models of autoimmune and inflammatory diseases. However, the exact nature of the stimulatory signals conferring regulatory properties to B cells is still not clear. We serendipitously observed that fluorescein isothiocyanate (FITC) binds to a significant proportion of naïve mouse B cells. Binding of FITC to the B-cell surface implicated at least in part the B-cell receptor. It triggered IL-10 production and allowed the endocytosis of FITC-coupled antigens followed by their presentation to CD4⁺ T cells. In particular, B cells incubated with FITC-OVA polarized OTII T cells towards a Tr1/Th2 phenotype *in vitro*. Further, the adoptive transfer of B cells incubated with FITC-labeled myelin oligodendrocyte glycoprotein peptide protected mice from experimental autoimmune encephalomyelitis, a T-cell-dependent autoimmune model. Together, the data show that FITC-stimulated B cells polarize immune responses towards Tr1/Th2 and acquire immuno-modulatory properties.

Keywords. Immuno-modulatory B cells, IL-10, experimental autoimmune encephalitis

Abbreviations. Bregs, regulatory B cells; EAE, experimental autoimmune encephalitis;

FITC, fluorescein isothiocyanate; BCR, B-cell receptor

Introduction

B cells with regulatory properties (Bregs) have been extensively studied over the past decade due to their therapeutic potential in the fields of autoimmunity and transplantation. Apart from therapeutic usage, Bregs may also exert pathogenic properties in human alloimmune and infectious diseases (1,2). In mice, regulatory properties have been attributed to phenotypically diverse B-cell sub-populations such as B1, marginal zone (MZ) and transitional 2 (T2) B cells (3–5). Various studies identified interleukin-10 (IL-10) as the critical effector of the regulatory potential of B cells. Indeed, B cells from IL-10-deficient mice are unable to protect against disease development. Apart from IL-10, Bregs express other immune-regulatory cytokines including IL-35 and transforming growth factor β (TGF- β) (6–8). Bregs can be induced following stimulation of CD40 (4), Toll-like receptors (TLRs) (3), CD80, CD86 or the B-cell receptor (BCR) (9). In several murine models, the mechanism of the Breg-dependent tolerance appears to be mainly antigen-specific (10–12). Yet, because of the lack of Bregs-specific surface markers and due to the scarcity of antigen-specific B cells in the circulation, the use of Bregs as effective agents for cell therapy remains to be made demonstrated. We have serendipitously observed that fluorescein isothiocyanate (FITC) binds to the BCR of a significant proportion of naive mouse B cells, and that the binding of FITC coupled to model antigens to the BCR triggers the endocytosis of the FITC-coupled antigen, and fosters the production of IL-10 by a substantial percentage of naive B cells. The B cells in turn influence the polarization of CD4 Th0 cells toward Th2/Tr1 cells. Here, we validated the immunoregulatory properties of FITC-stimulated B cells *in vitro* as well as *in vivo* using the model of experimental autoimmune encephalomyelitis (EAE).

Materials and methods

Animals, antigens and cell clones

Eight to 9-week-old BALB/c mice (males), C57BL/6 mice (females and males) and OTII mice (males) were obtained from Charles River (France). BCR-deficient V_H-LMP2A mice (males) (13) on BALB/c background, were provided by Dr Christophe Sirac (UMR CNRS 6101, Limoges, France). Inbred C57Bl6/J-exon 16 FIII-deficient male mice were grown bred at the pathogen-free animal facility of Cordeliers Research Center, Paris. All animal studies were performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (UPMC Paris) at the pathogen-free animal facility of Cordeliers Research Center, Paris. FITC-labeled and biotinylated MOG₃₅₋₅₅ peptides were purchased from PolyPeptide Laboratories (Strasbourg, France) and FITC-labeled ovalbumin (OVA) from Invitrogen (OVA-FITC, life technologies, Saint Aubin, France, contains 4 moles of FITC for 1 mole of OVA).

Ovalbumin (Sigma-Aldrich, Saint-Quentin Fallavier, France) was labeled with biotin using EZ-Link* NHS-LC-Biotin (Thermo scientific, Illkirch, France); the excess of free biotin was removed by dialysis against Phosphate-buffered saline (PBS). Cells were cultured in RPMI medium (Lonza, Levallois-Perret, France) supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin, 50 µM 2-ME, 0.1 mM non-essential amino acids and 10% of heat inactivated fetal bovine serum. DO54.8 is an OVA₃₂₃₋₃₃₉-specific mouse CD4⁺ T hybridoma restricted to I-A^d (C57BL/6).

Purification of B and T cells

B cells were purified from the spleen of BALB/c, V_H-LMP2A or C57BL/6 mice using either the “Pan B cell isolation kit” or CD19 MicroBeads (MACS, MiltenyiBiotec, Paris, France). Briefly, splenocytes were isolated after mechanical dilaceration of spleens of naïve mice and

splenocytes were incubated 90 s in ACK lysis buffer (life technologiesTM, Invitrogen) at room temperature. Cells were then passed through 70 μ m nylon membrane filters and suspended in PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin (BSA, Sigma-Aldrich) as recommended by the manufacturer. CD4⁺ T cells were purified from splenocytes of OTII and wild-type C57BL/6 mice using Dynabeads[®] Mouse CD4 (life technologyTM, Invitrogen). Total cells were also purified from the blood and lymph nodes of mice. In brief, blood cells were collected upon perfusion of the mice with 40 ml of 0.2 mM EDTA in PBS through the left ventricle. Draining lymph nodes were collected and mechanically dilacerated. Single cell suspensions were prepared as described above.

Binding and endocytosis tests

Binding of antigens to cell surface was evaluated using total or negatively selected B cells from splenocytes of naïve mice. Cells were incubated 1 hr on ice alone or in the presence of antigens. After incubation, cells were washed and stained with a PE-labeled anti-mouse CD19 antibody (1D3; BD biosciences, Le Pont de Claix, France) for 25 min at 4°C. Cells were then incubated for 25 min with APC-labeled annexin V (Invitrogen), washed and incubated with propidium iodide (Invitrogen) prior to FACS analysis. Binding of antigens was evaluated by flow cytometry (BD LSRII) either by direct fluorescence of FITC or using Pacific Blue-coupled streptavidin (Molecular Probes, Invitrogen) on the viable cell population (i.e., negative for annexin V and propidium iodide). Endocytosis of antigens was performed using negatively selected B cells purified as described above. B cells were incubated alone or in the presence of antigens for 1 hr at 4°C or 37°C. After incubation, B cells were washed, stained with a PE-labeled anti-mouse CD19 antibody (1D3; BD biosciences) for 25 min at 4°C, and permeabilized with the Cytotfix/Cytoperm kit (BD Biosciences, Le Pont de Claix, France). When indicated, the FITC and Pacific blue fluorescences were normalized using FITC-labeled and Pacific Blue-

labeled anti-CD19 IgG (clone 1D3). Endocytosis of antigens was evaluated by subtracting the normalized mean of fluorescence intensity (MFI) obtained at 37°C from that obtained at 4°C. Cells were acquired using a BD LSR II and analyzed with FACS Diva or FlowJo software (BD Biosciences).

Analysis of T-cell activation and polarization

The DO54.8 T-cell hybridoma ($2.5 \cdot 10^5$ cells) was incubated alone or with negatively selected B cells ($2.5 \cdot 10^5$ cells) from BALB/c mice in complete medium with or without FITC-labeled or biotinylated-OVA (10-200 µg/ml) for 2 days at 37°C. The supernatant was collected and the production of IL-2 by DO54.8 was quantified using a mouse IL-2 ELISA Set (BD OptEIA10™, BD bioscience). For T cell polarization, purified CD4⁺ T cells from OTII mice were labeled with CellTrace™ violet (CTV, life technology™, Invitrogen). Briefly, purified CD4⁺ T cells (10^6 cells/ml) were incubated with CTV (3 µM) for 20 min at 37°C in complete medium and washed to remove the excess of free dye. B cells (10^5 cells) from C57BL/6 mice were co-incubated with purified CD4⁺ T cells (10^5 cells) with or without OVA-FITC (5-20 µg/ml) for 6 days at 37°C. When indicated, B and T cells were incubated with a neutralizing anti-IL-10 antibody (JES3-19F, Biolegend, San Diego) or with a neutralizing anti-IL-10 receptor antibody (clone 1B1.2, a kind gift from Dr Sylvaine You (Institut Necker-Enfants Malades, Paris, France)). For intracellular cytokine staining, cells were stimulated *in vitro* with phorbol 12-myristate 13-acetate (50 ng/ml, Sigma-Aldrich) in the presence of Ionomycin (1 µg/ml, eBioscience, Paris, France) and Monensin (GolgiStop®, BD biosciences) for 6 hr at 37°C. T-cell polarization was evaluated using PE-Cy5-labeled anti-CD4 antibody (H129.19, BD biosciences), PE-labeled anti-IL-10 antibody (JES5-16E3, BD biosciences), APC-labeled anti-IFN γ antibody (XMG1.2, BD biosciences), Alexa 700-labeled anti-FoxP3 antibody (FJK-16s, eBioscience) and the FoxP3 staining buffer set (ebioscience) by flow cytometry.

Analysis of B-cell activation

Negatively selected B cells (10^6 /ml) from C57BL/6 mice were incubated alone, in the presence of $F(ab')_2$ fragments of an anti-mouse IgM antibody (10 μ g/ml, Jackson ImmunoResearch), of OVA-FITC (200 μ g/ml) or of OVA-biotin for 2 days at 37°C. B-cell activation was evaluated using Pacific blue-conjugated anti-CD19 (1D3), PE-conjugated anti-CD80 (16-10A1, BD biosciences), APC-conjugated anti-CD83 (Michel-19, BD biosciences), PE-conjugated anti-I-Ab/I-Eb(2G9, BD biosciences) and biotinylated anti-CD86 (GL1, BD biosciences) associated with the Alexa-fluor® 700 labeled streptavidin (Molecular probes®, Invitrogen). Briefly, 10^6 B cells on day 2 were washed and suspended in 100 μ l of ice-cold PBS containing 0.5% BSA and secondary antibodies (2 μ g/ml final concentration of each) for 25 min on ice. B cells were then washed and suspended in 300 μ l of PBS/0.5% BSA supplemented with propidium iodide (5 ng/ml). The level of expression of surface was evaluated by flow cytometry with FACS Diva software.

Analysis of B-cell subpopulations.

Total splenocytes were isolated from naïve mice as previously described. Cells were surface stained with a combination of PE-labeled anti-CD21/CD35 antibody (4E3, ebioscience), PE-Cy7-labeled anti-CD23 antibody (B3B4, ebioscience), APC-labeled anti-CD19 antibody (1D3), and PE-labeled anti-CD24 antibody (M1/69, BD Pharmingen) associated with the Alexa-fluor® 700-labeled streptavidin (Molecular probes®, Invitrogen). The marginal zone B cells (MZ) were referred as $CD24^- CD21^{++} CD23^{low}$, the follicular B cells (FO) as $CD24^- CD21^{low} CD23^+$, the transitional 1 B cells (T1) as $CD24^+ CD21^- CD23^-$ and the transitional 2 B cells (T2) as $CD24^+ CD21^+ CD23^+$.

Cytokine production by B cells

Purified B cells were obtained as described above. B cells (10^6 cells/ml) were incubated alone

or in the presence of antigens (200 µg/ml) for 2 days at 37°C. Supernatant was stored at -80°C and B cells were stimulated *in vitro* for intracellular cytokine staining as previously described. Intracellular staining was evaluated using APC-labeled anti-CD19 antibody (clone 1D3), PE-labeled anti-IL-10 antibody (JES5-16E3, BD biosciences) and the Cytotfix/Cytoperm kit (BD Biosciences) by flow cytometry. Quantification of cytokines secreted by B cells in the supernatant was assessed using the BD *CBA Mouse Inflammation Kit* (BD biosciences).

Induction of EAE

Female C57BL/6 mice were immunized with 100 µg MOG₃₅₋₅₅ peptide emulsified in complete Freund's adjuvant (CFA, Sigma-Aldrich, v/v 1:1) containing 400 µg of *Mycobacterium tuberculosis* (Difco Laboratories, L'Arbresle, France). A final volume of 200 µl was injected subcutaneously at 2 sites over the flanks. In addition, 150 ng of Pertussis toxin (List Biologic Laboratories, Meudon, France) was given intravenously on the same day and 2 days later. Clinical signs of EAE were assessed daily by the following scoring system: 0, no sign; 1, hindlimb weakness; 2, hindlimb weakness and tail paralysis; 3, hindlimb and tail paralysis; 4, hindlimb and tail paralysis and forelimb weakness; 5, moribund.

B-cell transfer experiments

On day five and day one before EAE induction, as well as on day five after induction of the disease, mice received intravenously B cells (10⁶ cells) from naïve animals incubated alone or in the presence of FITC-labeled or biotinylated antigens for 3 hr at 37°C in HL-1 medium (Lonza).

Statistics

All data are shown as mean ± SEM (n between 3 and 10, as indicated). Statistical significances

were assessed using the double-sided non-parametric Mann Whitney U test. We used the two-way ANOVA with Bonferroni's post-hoc t test for comparing EAE scores (* $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$).

Results

B cells from naïve mice bind FITC-labeled protein in a BCR-dependent manner

We first investigated the capacity of splenic B cells from naïve wild-type mice to bind FITC. To this end, we used FITC coupled to OVA as a carrier protein. FITC-labeled OVA (200 µg/ml) was incubated for 1 hr at 4°C with splenocytes purified from naïve C57Bl/6 mice. Binding was analyzed by flow cytometry. Since binding of FITC at the surface of B lymphocytes has been documented previously (14), the usage of FITC-labelled isotype IgG was not conceivable to set thresholds; the threshold of FITC positivity was therefore based on the CD19-negative population. Under such conditions, OVA-FITC-positive B cells represented $9.9\pm 1.3\%$ (mean \pm SEM) of the total splenocytes (Figure 1A) and $16.4\pm 2.26\%$ of the CD19⁺ cells. Accordingly, the mean fluorescence intensities of FITC among the CD19⁺ and CD19⁻ populations were 263 ± 21.7 and 84 ± 10 , respectively. Binding of OVA-FITC to the surface of purified CD19⁺ cells increased in a dose-dependent manner and reached a plateau of >25% positive cells at 800 µg/ml (Figure 1B). Interestingly, biotinylated OVA failed to bind the surface of CD19⁺ cells, indicating that binding is mediated by the FITC moiety rather than OVA. Importantly, 15 to 25% of purified CD19⁺ cells bound FITC-coupled MOG₃₅₋₅₅ peptide, as well as FITC-coupled factor IX and FITC-coupled factor VIII (Figure 1C, all at 4 µM), while no binding was observed with the biotinylated forms of the molecules. This indicates that the nature of the antigens does not influence the FITC-mediated antigen binding on the B-cell surface. The binding of FITC to B cells was mediated at least in part by the BCR as shown by the absence of OVA-FITC binding to B cells isolated from V_H-LMP2A BCR-deficient (BCR^{-/-}) BALB/c mice (13), when compared to B cells from naïve wild-type BALB/c mice ($20.3\pm 1.7\%$) (Figure 1D).

We then investigated whether the binding of OVA-FITC by CD19⁺ cells is related to the natural broad reactivity of some B-cell sub-populations. Different percentages of inguinal lymph nodes

and peritoneal B cells bound OVA-FITC ($21.1\pm 2.2\%$ and $60.8\pm 4.7\%$, respectively, Figure 2A). Similarly, the different splenic B-cell subsets, T1, FO, T2 and MZ B cells, bound OVA-FITC to different extents (16.2 ± 1.9 , 16.8 ± 1.8 , 31.5 ± 6.4 and $29\pm 4.5\%$, respectively, Figures 2C and 2D). Interestingly, the levels of expression of surface IgM by the different splenic B cell subsets, correlated positively with the intensity of binding of OVA-FITC (Figures 2E and 2F). All together, these results suggest that the binding of the FITC moiety by the B cells is not restricted to a specific subset of B cells but is strongly related to the level of immunoglobulin expression on the B cell surface.

FITC-labeled OVA is endocytosed and presented by naive splenic B cells

We then explored the consequences of FITC binding to the BCR of naive B cells on their antigen presentation capacity. Purified naive B cells endocytosed FITC-labeled OVA in a dose-dependent manner, but failed to endocytose biotin-labeled OVA (Figure 3A). Co-incubation of purified splenic B cells from BALB/c mice with the OVA₃₂₃₋₃₃₉-specific CD4⁺ DO54.8 T cells hybridoma in the presence of OVA-FITC, lead to a dose-dependent activation of DO54.8 T cells (Figure 3B). T cells incubated with B cells alone, with biotinylated-OVA (Figure 3B), or incubated with OVA-FITC in the absence of B cells, failed to secrete IL-2.

Binding of FITC-labeled OVA to B cells induces IL-10 production in vitro

We then evaluated and compared the cytokines secreted by purified splenic B cells incubated either with a BCR cross linker (anti-IgM F(ab')₂ fragment) or with OVA-FITC *in vitro*. When stimulated for 24 hr with anti-IgM F(ab')₂ fragments, purified splenic B cells secreted high amounts of TNF α and MCP-1 as compared to control B cells (Figure 4A). In contrast, B cells stimulated with OVA-FITC (200 μ g/ml) secreted high amounts of IL-10 as compared to cells incubated alone, with anti-IgM F(ab')₂ fragments or with OVA-biotin. Because stimulation

with OVA-FITC triggered the production of IL-10 by B cells, we investigated whether the binding of OVA-FITC is restricted to previously described population of IL-10 secreting regulatory B cells expressing high level of CD1d and CD5 markers (5). OVA-FITC-binding B cells belonged to both the CD19⁺CD1d^{hi}CD5⁺ and CD19⁺CD1d^{low}CD5⁻ cell-subpopulations (Supplementary Figure 1). OVA-FITC-binding CD19⁺CD1d^{hi}CD5⁺ cells however accounted for 0.56±0.29% of the total splenic OVA-FITC-positive B cells.

We then compared the activation markers expressed by B cells stimulated by anti-IgM F(ab')₂ fragments or OVA-FITC. Naive B cells incubated with anti-IgM F(ab')₂ fragments underwent strong activation which was not the case when B cells were incubated with OVA-FITC (Figure 4B and Supplementary Figure 2), as evidenced by the percentage of cells expressing CD86 (75.8±9.3 versus 24.1±3.9%, respectively, p=0.003), CD83 (66.5±3.9 versus 2.7±0.9%, respectively, p=0.0004) and CD19^{high} (18.8±2.2 versus 9.3±0.9%, respectively, p=0.02). Likewise, the MFI of HLA class II was drastically increased upon B cell stimulation with anti-IgM F(ab')₂ fragments (44.7x10³±8.5x10³) as compared to OVA-FITC (11x10³±3.4x10³, p=0.01). The incubation of B cells alone or in presence of OVA-biotin failed to induce B-cell activation (Figure 4B).

Presentation of FITC-labeled OVA by naïve splenic B cells polarizes CD4⁺ T cells toward Th2/Tr1 cells

We explored the consequences of BCR-mediated FITC binding to the BCR of naive B cells on their antigen presenting capacity to polarize the CD4⁺ T cell response. The proliferation and polarization of primary OVA₃₂₃₋₃₃₉-specific CD4⁺ T cells following incubation with negatively selected OVA-FITC-stimulated B cells was assessed using naive CD4⁺ splenic T cells purified from OTII mice. Incubation of CD4⁺ OTII T cells with ConA, used as a positive control, induced the proliferation of 38.2±1.7% of the total CD4⁺ T cells (Figure 3C, upper panels). The

presence of OVA-FITC increased in a dose-dependent manner the percentage of proliferating T cells (up to $27.8 \pm 0.98\%$). In the presence of ConA, $0.6 \pm 0.1\%$ and $2.3 \pm 0.2\%$ of the $CD4^+$ OTII T cells were positive for intracellular $IFN\gamma$ and IL-10, respectively (Figure 3C, bottom panels), while most of the OTII T cells incubated alone were negative for $IFN\gamma$ and IL-10. The co-culture of OTII $CD4^+$ T cells and C57BL/6 B cells in the presence of OVA-FITC lead to a dose-dependent generation of IL-10⁺ $CD4^+$ T cells, and a marginal increase in percentages of $IFN\gamma^+$ $CD4^+$ T cells. Importantly, there was no generation of FoxP3-positive $CD4^+$ T cells. In the presence of ConA, both dividing (CTV^{low}) and non-dividing (CTV^{high}) OTII T cells were negative for IL-10 expression (Figure 3D). In the presence of C57BL/6 B cells and OVA-FITC, the percentages of IL-10⁺ $CD4^+$ OTII T cells increased in a dose-dependent manner up to $36.8 \pm 2.8\%$ and $16.1 \pm 1.4\%$ for dividing and non-dividing OTII T cells, respectively (Figure 3D).

Adoptive transfer of MOG₃₅₋₅₅-FITC-loaded naive B cells protects mice from EAE

We then investigated the protective potential of adoptively transferred naive splenic B cells pre-incubated with FITC-labeled MOG₃₅₋₅₅ peptide in EAE, an experimental model of multiple sclerosis. Recipient mice received purified splenic B cells (10^6 cells) from naive mice pre-incubated alone, with biotin-MOG₃₅₋₅₅, with FITC-MOG₃₅₋₅₅, or with OVA-FITC. Following induction of EAE, mice injected with untouched purified B cells or with B cells incubated with MOG-biotin developed the first clinical signs at day 10-11, with a peak of the disease at day 17 and with a mean clinical score of 2.85 ± 0.38 and 2.89 ± 0.27 , respectively (Figure 5A). Mice injected with B cells incubated with OVA-FITC exhibited a delayed onset of the disease (day 19) but the severity remained comparable to that of the control group with a mean clinical score of 2.85 ± 0.38 (Figure 5A). The transfer of B cells pre-incubated with MOG₃₅₋₅₅-FITC not only delayed the onset of the disease (day 14), but also significantly decreased the severity of the

disease (mean of clinical score of 1.22 ± 0.31 at day 18). At day 18, representative mice that received B cells pre-incubated with FITC- or with biotin-labeled MOG₃₅₋₅₅ were sacrificed and cells from the spleen, blood and draining lymph nodes (DLN) were isolated. Percentages of IL-10-positive CD4⁺ T cells (Figure 5B) increased significantly in the blood, DLN and spleen of mice adoptively transferred with naive B cells pre-incubated with MOG₃₅₋₅₅-FITC (Figure 5B). Analysis of the IL-10⁺CD4⁺ T-cell populations for the expression of FoxP3 showed no statistical difference in any of the tested organs between the 2 groups of mice, in agreement with *in vitro* data. The percentage of IFN γ ⁺CD4⁺ T cells was marginally although statistically higher (P=0.042) in the DLN of mice that received B cells pre-incubated with MOG₃₅₋₅₅-FITC.

Discussion

Our results indicate that stimulation of naïve B cells by protein-coupled FITC polarizes B cells towards an immunoregulatory phenotype. Notably, the adoptive transfer of naïve B cells stimulated by MOG-FITC reduces disease severity in EAE. Several pieces of evidence suggest that FITC is a promiscuous BCR ligand. Incubation of OVA-FITC, used as a model antigen, with total splenic B cells from naïve mice resulted in a 3-fold increase in mean fluorescent intensity of the B-cell population as compared to the CD19-negative population. Besides, FITC-labeled antigens failed to bind to the surface of B lymphocytes from BCR^{-/-} V_H-LMP2A mice, suggesting that the binding to B cells is mediated by the BCR complex. Several approaches have been described to generate Bregs, wherein the involvement of the BCR is only indirectly suggested. In the arthritis model of DBA/1 mice immunized with collagen, IL-10⁺ B cells were generated *ex vivo* only provided that the B cells originate from immunized animals and are stimulated with an anti-CD40 antibody in the presence of the antigen (4). Moreover, in the case CD1d^{high}CD5⁺ B cells (15), stimulation with LPS, a recognized BCR ligand (16), was able to induce IL-10 production by B cells from both wild-type and MyD88-deficient mice. Of note, BCR diversity (i.e., polyclonality) was demonstrated to be required for B cells to produce IL-10 upon LPS stimulation (15), also inferring that not all B cells are sensitive to IL-10 induction, and thus indirectly suggesting the implication of BCR signaling as a pre-requisite for IL-10 production. In addition, impeding in the BCR-mediated calcium influx exclusively on B cells resulted in a reduced IL-10 production following BCR and anti-CD40 stimulation (17). More recently, direct evidences in a mouse allograft tolerance and during RSV infection in the human, further emphasized the importance of BCR stimulation in the acquisition of immune-regulatory properties by B cells (1,10).

B cells stimulated with OVA-coupled FITC acquired a phenotype characterized by low expression of activation markers, by the low production of TNF α and elevated production of

IL-10, and by antigen presentation to CD4⁺ T cells. Such a phenotype is reminiscent of the tolerogenic phenotype of immature dendritic cells (18,19). FITC is a small polyaromatic compound that has no known relevance in physiology. Even if the molecular mechanisms explaining the natural reactivity of B cells toward FITC remain unclear, the binding of circulating immunoglobulins and BCR to aromatic compounds and to fluorochromes has already been described (20–23). Incidentally, the capacity of FITC to bind to B lymphocytes from naive mice or from the peripheral blood of healthy donors has been described previously, without a demonstration of the implication of the BCR in the phenomenon (14,24).

The nature of the B-cell population(s) targeted by aromatic compounds, and in particular by FITC, remains unclear. Up to 15 to 20% of the total splenic B cells bound FITC, suggesting that the binding is not restricted to a single set of V_H and V_L genes. Besides, all B-cell subpopulations in the spleen, as well as B cells from the lymph nodes and from the peritoneum were capable of binding FITC, with a preference for peritoneal B1 cells, and splenic T2 and MZ B cells. Altogether, our data demonstrate that FITC-binding is not restricted to a subset of splenic B cells and its intensity strongly dependent on the density of BCR expressed on the surface of the cell. Because FITC binding to BCR confers the target B cells with immunoregulatory properties, our data indirectly suggest that Bregs (at least defined by the capacity to produce IL-10) do not belong to a distinct subset of B cells. Conversely, any B cell may polarize towards an IL-10 producing B cell provided the presence of appropriate signals. OVA is widely used in several animal models may be contaminated by LPS, a potent IL-10 inducer in B cells (25). Importantly, BCR^{-/-} B cells from V_H-LMP2A mice failed to produce IL-10 following stimulation OVA-FITC. We cannot however rule out the possibility that both BCR and TLR4 stimulations are necessary for IL-10 production by B cells. OVA-FITC stimulation of B cells from MyD88/TRIF double knockout mice would help to understand the possible involvement

of the TLR4 signaling in the production of IL-10 by splenic B cells from WT mice. The complete nature of these signals remains to be deciphered.

The protective role of Bregs has been exploited experimentally in a large array of experimental models for Th1-mediated autoimmune diseases (4,5,9,15,26–29). In the human, neonatal nBregs downregulated Th1 cell responses against RSV in an IL-10-dependent manner (1). Conventional Bregs have been shown to convert effector T cells into IL-10-producing T cells through the provision of IL-10 (26,29). Here, the adoptive transfer of MOG₃₅₋₅₅-FITC “educated” splenic B cells from naïve mice, reduced the severity of EAE in recipient mice. It was associated with an increased percentage of CD4⁺IL-10⁺FoxP3⁻ T cells reminiscent of the phenotype of the Tr1 or Th2 T-cell subsets. Conversely, the transfer of B cells “educated” with OVA-coupled FITC, that do produce IL-10 *in vitro*, failed to efficiently protect mice from EAE, highlighting the antigen-dependency of the conferred protection and suggesting the recruitment of endogenous MOG₃₅₋₅₅-specific CD4⁺ T by MOG₃₅₋₅₅-FITC “educated” Bregs. These results are in line with seminal works in the Breg field describing the importance of antigen specificity for the regulatory efficacy of IL-10-producing B cells in autoimmune diseases, allograft resistance models and infectious diseases (1,4,10). Taken together, our results pave the way towards the *ex vivo* generation of antigen-specific immuno-modulatory B cells using FITC-coupled proteins. Induced antigen-specific Bregs should represent novel therapeutic tools for the treatment of Th1-mediated inflammatory diseases.

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Authors contributions

Planned the work: CP, AP, JB, SVK, JDD, SLD

Performed experiments: CP, JR, SD, SP

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The authors declare no conflict of interest

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Figures legends

Figure 1. Naïve B cells bind FITC in a BCR-dependent manner. Total cells (10^6 cells/ml) from the spleen (A) of naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 μ g/ml, OVA-FITC) on ice for 1 hr in serum free medium. (B and C) Purified B cells from naïve C57BL/6 mice were incubated with FITC-labeled or biotinylated-antigens (0 to 800 μ g/ml in B, or 200 μ g/ml in C) on ice for 1 hr in serum free medium. (D) Purified B cells from naïve BALB/c mice or naïve BCR^{-/-} mice (V_H-LMP2A mice) were incubated alone or in presence of OVA-FITC (200 μ g/ml) for 1h at 4°C. The graphs depict the percentage of viable antigen-positive cells evaluated either by the direct fluorescence of the FITC moiety or using Pacific Blue-labeled streptavidin by flow cytometry (cell viability was >85%). Percentage of positive cells from at least three independent experiments is presented. Error bars represent SEM.

Figure 2. FITC binds to different B-cell subsets. Total cells (10^6 cells/ml) from the spleen (Spl), the lymph nodes (LN) or the peritoneum (Perit) of naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 μ g/ml, OVA-FITC) on ice for 1 hr in serum free medium. (A). Splenic transitional 1 and 2 (T1 and T2), the marginal zone (MZ) and the follicular (FO) B cells were identified according to the level of expression of the surface markers CD19, CD21, CD23 and CD24 (B and C). The percentages of cells binding OVA-FITC and the intensities of OVA-FITC binding to each B-cell subset were evaluated by the direct fluorescence of the FITC moiety by flow cytometry (cell viability was >85%) (D). The intensity of BCR expression by each B-cell subset was also evaluated (E) and correlated with the intensity of OVA-FITC binding (F). Error bars represent SEM for three mice.

Figure 3. Natural FITC-binding naive B cells endocytose FITC-labeled OVA and induce IL-10-producing CD4⁺ T cells. (A) Purified splenic B cells (10⁶ cells/ml) from naive C57BL/6 mice were incubated with FITC-labeled (full squares) or biotinylated-OVA (empty circles) at 0 to 800 µg/ml, for 1 hr on ice or at 37°C in serum free medium. Endocytosis of OVA was evaluated either by direct fluorescence of FITC or using Pacific Blue-labeled streptavidin on fixed and permeabilized cells by flow cytometry. The graph depicts the difference of mean fluorescence intensity between 37°C and 4°C. The MFI was normalized with respect to FITC-labeled anti-CD19 antibody or Pacific Blue-labeled anti-CD19 antibody, used as positive controls. (B) Purified splenic B cells (2.10⁵ cells) from naïve BALB/c mice were co-incubated with the OVA₃₂₃₋₃₃₉-specific hybridoma (DO54.8, 2.10⁵ cells) alone, in the presence of FITC-labeled (full bars) or biotinylated-OVA (empty bars) at 10 to 200 µg/ml for 48 hr at 37°C in complete medium. The graph depicts the concentration of IL-2 secreted by DO54.8 in the supernatant evaluated by ELISA. The statistical significance of the observed differences between B cells incubated with OVA-FITC or OVA-biotin was determined by the double-sided non-parametric Mann-Whitney U test: ***p*<0.01 and ****p*<0.001. (C and D) Negatively selected splenic CD4⁺ T cells (1.5x10⁵ cells) from OTII C57BL/6 mice were stained with CTV and co-incubated with purified splenic B cells (1.5x10⁵ cells) from naive C57BL/6 mice alone or in the presence of OVA-FITC (5-20 µg/ml) for 6 days at 37°C in complete medium. As a positive control, CD4⁺ T cells were incubated with 2 µg/ml of Concanavalin A (ConA). Cells were then washed and stimulated for 6 hr at 37°C in the presence of phorbol 12-myristate 13-acetate (50 ng/ml), ionomycin (1 µg/ml) and Monensin. The proliferation (C, top panels) and polarization (C, bottom panels; D) of CTV-stained CD4⁺ T cells were evaluated on fixed permeabilized cells by flow cytometry. Values in quadrants depict the percentages of cells positive for the different markers: CTV, IFN-gamma and/or IL-10, gated on the CD4-positive

population. Data in all panels are representative of three independent experiments. Error bars represent SEM.

Figure 4. Stimulation of naïve B cells by OVA-FITC leads to the production of IL-10. (A) Negatively selected splenic B cells (10^6 cells/ml) from naïve C57BL/6 mice were incubated alone, with F(ab')₂ fragments of an anti-IgM antibody (10 µg/ml), with FITC-labeled or biotinylated-OVA (200 µg/ml) for 2 days at 37°C in complete medium. The graphs represent the concentrations of TNFα, IL-10 and MCP-1 secreted by the B cells in the supernatant. Data are representative of three independent experiments. (B) The activation status of negatively selected splenic B cells (10^6 cells/ml) from naïve C57BL/6 mice incubated for 2 days alone, with F(ab')₂ fragments of an anti-IgM antibody (10 µg/ml), with FITC-labeled or biotinylated-OVA (200 µg/ml) was assessed by the surface expression levels of CD80, CD86, CD83, CD19 and of MHC-II. Data are representative of two independent experiments. Error bars represent SEM. Statistical significance was assessed using ANOVA test: ns: not-significant; *: $p < 0.05$ and **: $p < 0.01$.

Figure 5. Adoptive transfer of MOG₃₅₋₅₅-FITC-loaded naïve B cells protects mice from EAE. (A) EAE was induced in 8 to 9 week-old female wild-type C57BL/6 mice by immunization with MOG in Freund's adjuvant and injection of Pertussis toxin. EAE mice were intravenously administered with negatively selected splenic B cells from naïve C57BL/6 mice (10^6 B cells/mouse) five days and one day before induction of EAE, as well as 5 days after induction of EAE. Mice (10 mice/group) received B cells that had been incubated alone (full circles), with OVA-FITC (200 µg/ml, empty squares), with biotinylated (4 µM, empty circles) or FITC-labeled MOG₃₅₋₅₅ (4 µM, full squares), for 3 hr at 37°C in serum free medium. The graph depicts the mean clinical scores over a period of 30 days. Error bars represent SEM (n=10 mice

per group). Statistical significance of differences in EAE scores between groups was analyzed by the two-way ANOVA with Bonferroni's post-hoc t test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. (B) Mice that received MOG₃₅₋₅₅-biotin loaded-B cells (empty circles) or MOG₃₅₋₅₅-FITC loaded-B cells (full squares) were sacrificed at day 18 after induction of EAE. The graphs depict percentages of CD4⁺ T cells from blood, draining lymph nodes (DLN) and spleen. The studied T-cell populations include CD4⁺ T cells that are positive for IL-10 or for IFN γ , and CD4⁺IL-10⁺ T cells that are positive for FoxP3 expression. Horizontal lines depict mean values and SEM. Statistical significance was assessed using the double-sided non-parametric Mann Whitney U test: ns: not-significant; *: $p < 0.05$. Data are representative of 2 independent experiments.

Supplementary material

Supplementary Figure 1. Both C19⁺CD1d^{hi} CD5⁺ and C19⁺CD1d^{low}CD5⁻ cells may bind FITC-labeled OVA. Total splenocytes (10⁶ cells/ml) from naïve C57BL/6 mice were incubated alone or in presence of FITC-labeled ovalbumin (200 µg/ml, OVA-FITC) on ice for 1 hr in serum free medium. Percentages of OVA-FITC positive B cells were measured on splenic C19⁺CD1d^{hi} CD5⁺ and C19⁺CD1d^{low}CD5⁻ B cells, using the direct fluorescence of the FITC moiety of viable cells by flow cytometry (cell viability was >85%). Data represent means±SEM after measurements performed on 3 individuals. Statistical significances were assessed using the double-sided non-parametric Mann-Whitney U test: ** $p < 0.01$; **** $p < 0.0001$.

Supplementary Figure 2. Absence of activation of naïve B cells by OVA-FITC. The activation status of negatively selected splenic B cells (10⁶ cells/ml) from naïve C57BL/6 mice incubated for 2 days alone, with F(ab')₂ fragments of an anti-IgM antibody (10 µg/ml), with FITC-labeled or biotinylated-OVA (200 µg/ml) was assessed by the surface expression levels of CD80, CD86, CD83, CD19 and of MHC-II. The graphs depict histograms of raw flow cytometry data for one representative experiments. Values indicated on histograms represent % of cells positive for CD19, CD80, CD83 and CD86. In the case of MHCII, mean fluorescent intensities (MFI) are reported.

Figure 1

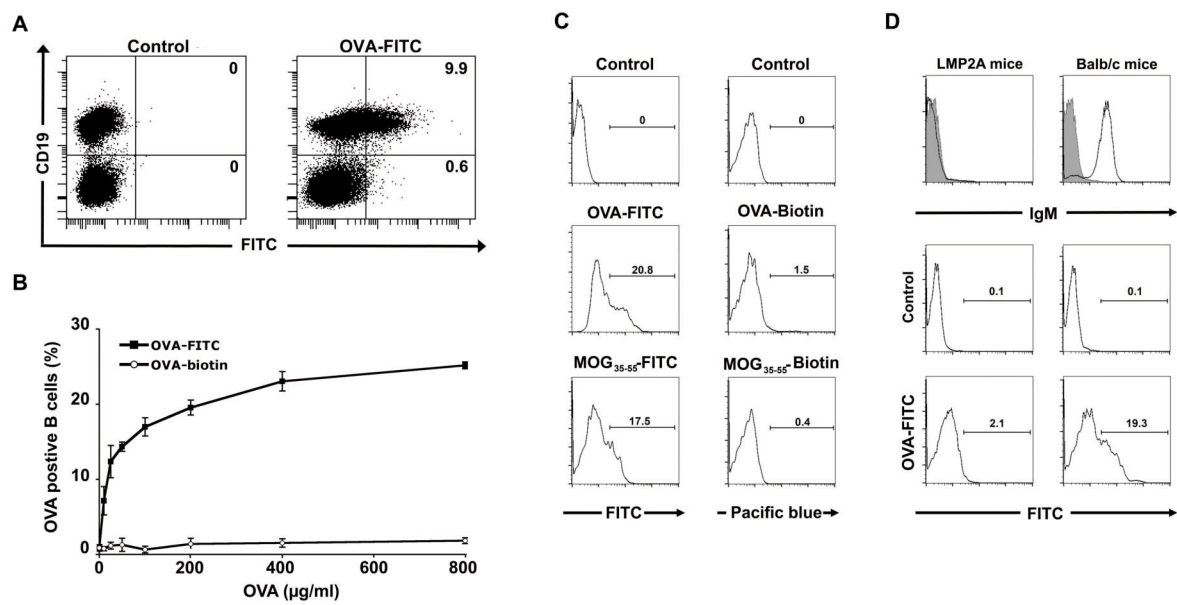


Figure 2

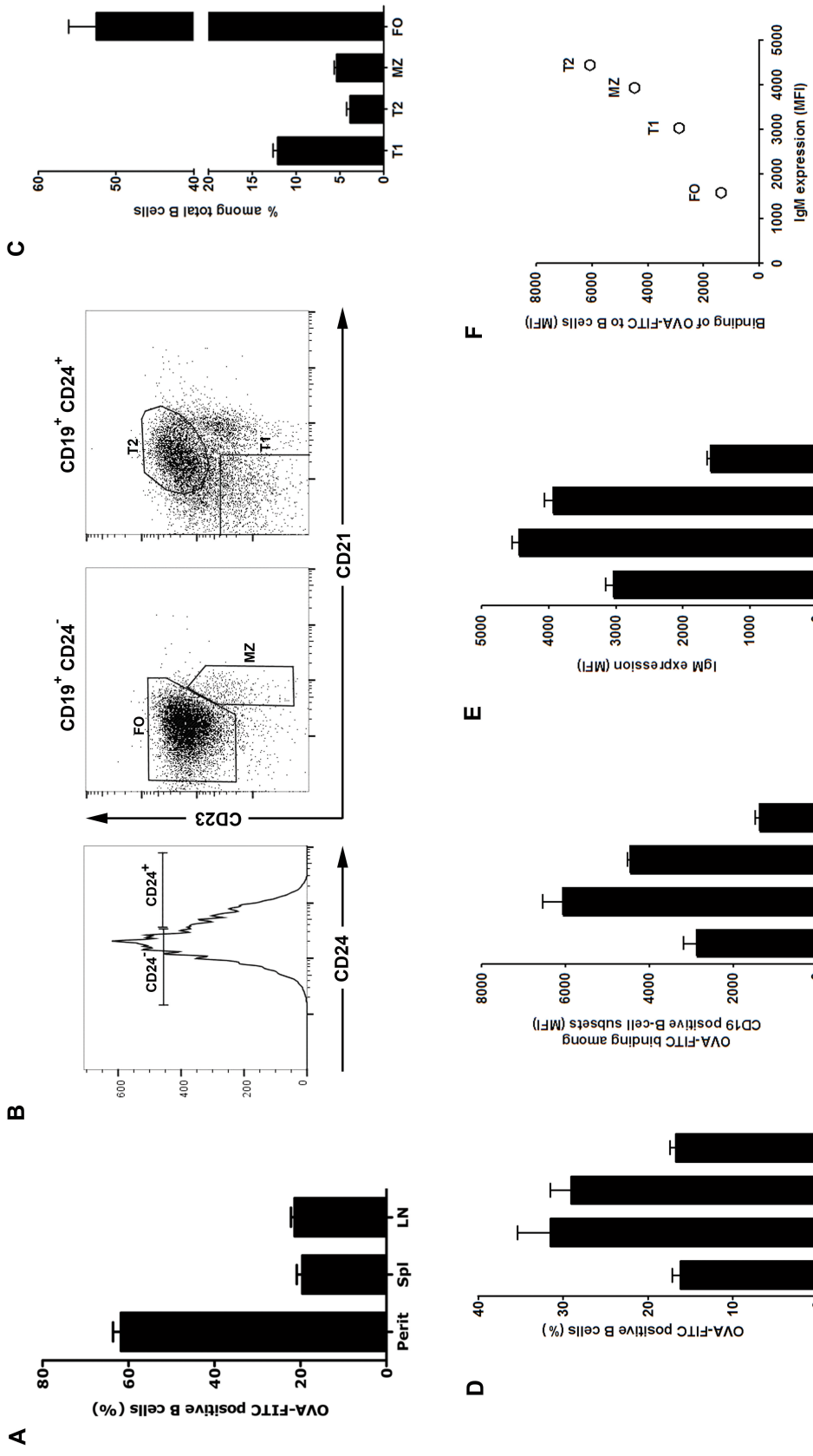


Figure 3

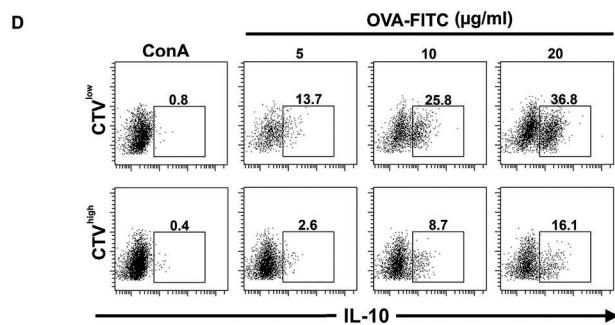
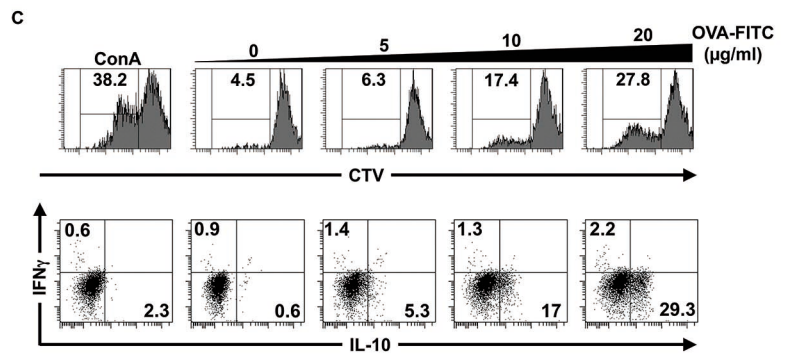
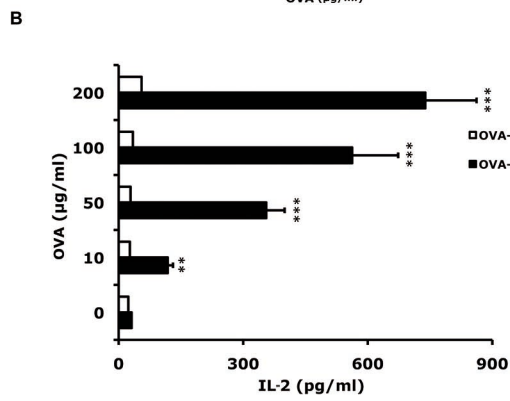
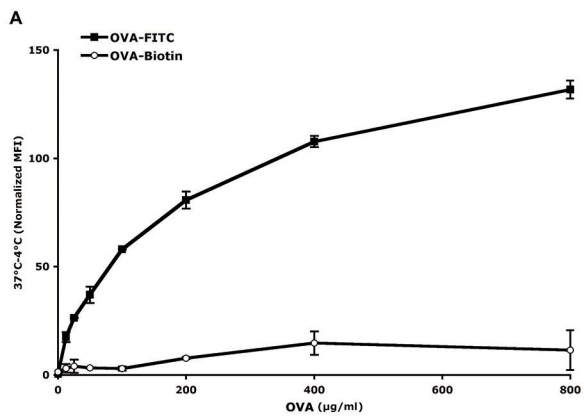


Figure 4

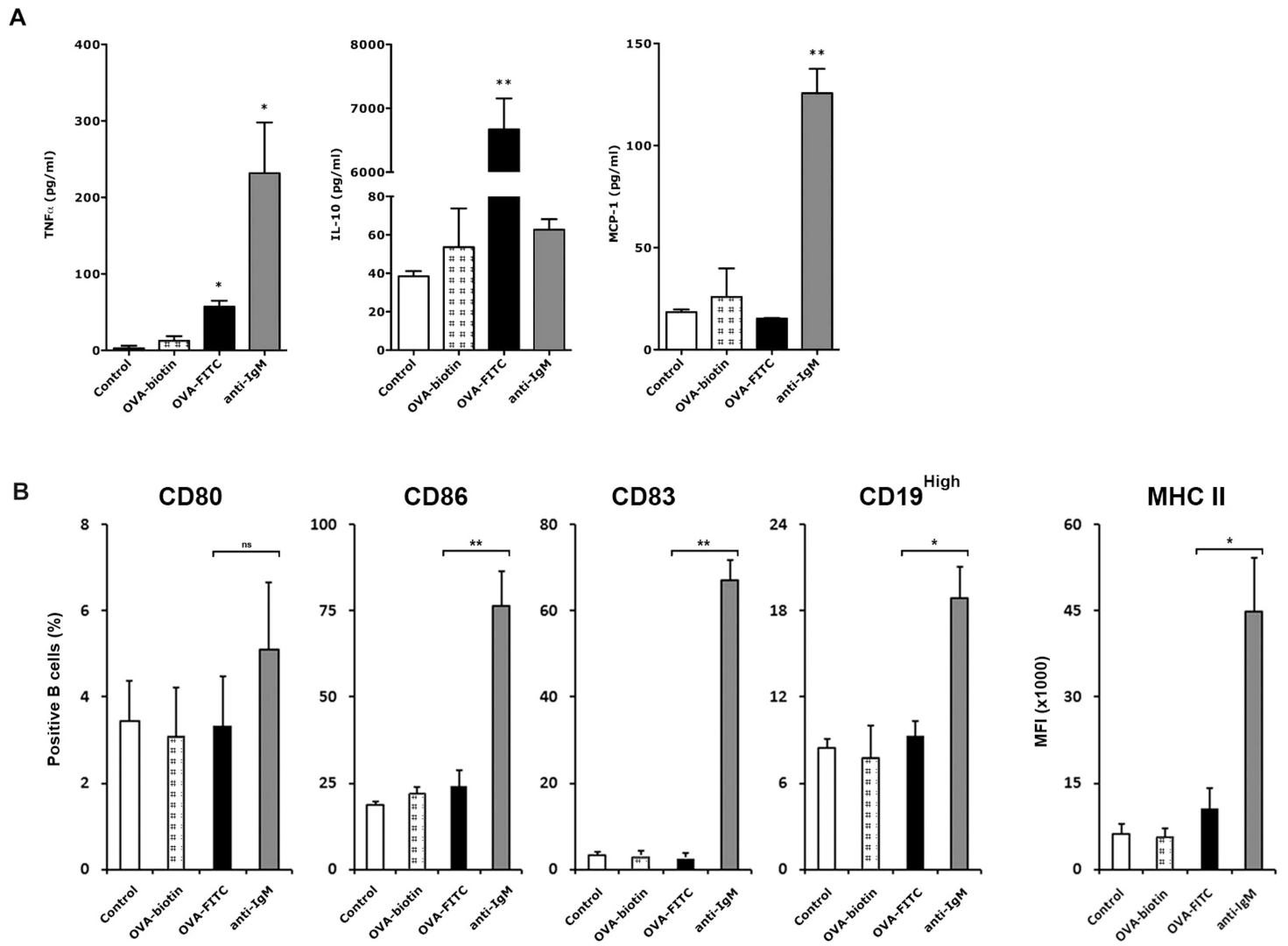
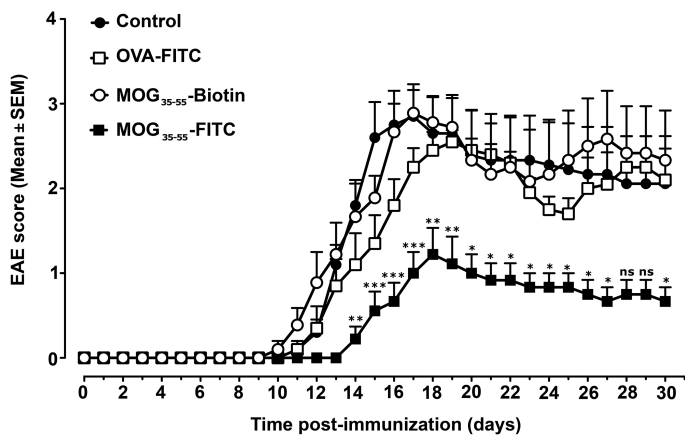
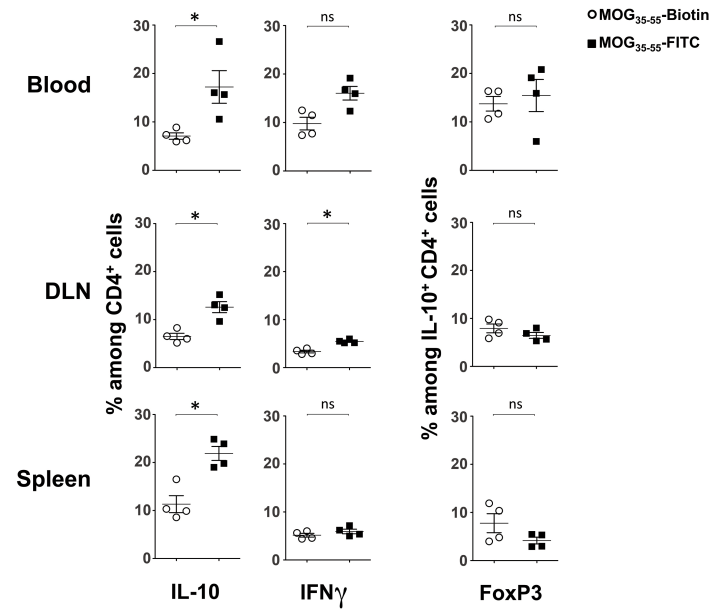


Figure 5

A



B





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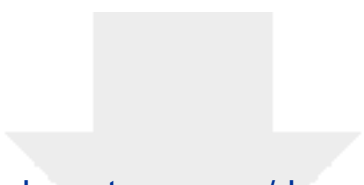
The authors declare that they have no conflict of interest related to the present work and that the work is not submitted for publication elsewhere.

CP, SVK, JDD and SLD are inventors of a patent entitled "Method for obtaining a population of interleukin 10-secreting B cells" n° EP13305783 that has been withdrawn.



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