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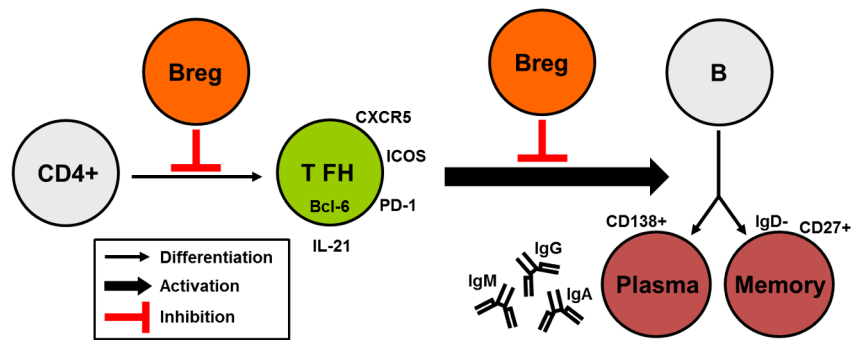
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Human regulatory B cells control Tfh response

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ABSTRACT

Background: Follicular helper T (Tfh) cells support the terminal B-cell differentiation. Human regulatory B (Breg) cells modulate cellular responses but their control of Tfh-dependent humoral immune responses is unknown.

Objective: To assess the role of Breg cells on Tfh development and function.

Methods: Human T cells were polyclonally stimulated in the presence of IL-12 and IL-21 to generate Tfh cells. They were co-cultured with B cells to induce their terminal differentiation. Breg cells were included in these cultures and their effects evaluated using flow cytometry and ELISA.

Results: Bcl-6, IL-21, ICOS, CXCR5 and PD-1 expressions increased on stimulated human T cells, characterizing Tfh-cell maturation. In co-cultures, they differentiated B cells into CD138⁺ plasma and IgD⁻CD27⁺ memory cells, and triggered immunoglobulin secretions. Breg cells obtained by TLR9 and CD40 activation of B cells, prevented Tfh-cell development. Added to Tfh and B cells co-cultures, they inhibited the B-cell differentiation, impeded immunoglobulin secretions, and expanded Foxp3⁺CXCR5⁺PD-1⁺ follicular regulatory T (Tfr) cells. Breg cells modulated IL-21R expressions on Tfh cells and B cells, their suppressive activities involved CD40, CD80, CD86, and ICAM interactions and required production of IL-10 and TGFβ.

Conclusion: Human Breg cells control the Tfh-cell maturation, expand Tfr cells and inhibit the Tfh-mediated antibody secretion. These novel observations demonstrate a role for the Breg in the germinal center reactions and suggest that deficient activities may impair the Tfh-dependent control of humoral immunity and may lead to the development of aberrant autoimmune responses.

KEY MESSAGES:

Human Tfh cells can be induced in vitro and trigger the terminal differentiation of B cells.

Human regulatory B cells suppress Tfh cell development and function.

Impaired regulatory B cell activities may contribute to the development of aberrant autoimmune responses.

CAPSULE SUMMARY

Our study describes *in vitro* models assessing the role of human regulatory B cells to modulate Tfh cell development and function and hence, to control the humoral immune response.

KEY WORDS:

Regulatory B cells, follicular T helper cells, IL-12, IL-21, antibody production, humoral immune response.

ABBREVIATIONS

Ab	Antibody
Bcl-6	B cell lymphoma 6
Breg	Regulatory B
CXCR5	Chemokine receptor 5
DC	Dendritic cells
DCs	Dendritic cells
GC	Germinal centers
GCs	Germinal centers
HD	Healthy donors
ICOS	Inducible costimulator
SLE	Systemic lupus erythematosus
Tfh	Follicular T helper
Tfr	Follicular regulatory T
TGF	Transforming Growth Factor

INTRODUCTION

Antibody (Ab)-producing cells and memory B cells are terminally differentiated B cells that arise during adaptive humoral immune responses in germinal centers (GCs),¹ under the supervision of T follicular helper (Tfh) cells.² The development and control of Tfh cells is still a matter of debate.³ One day after priming by dendritic cells (DCs) in the T zone, antigen-activated CD4⁺ T cells up-regulate Bcl-6 which is necessary for Tfh cell generation.⁴ This initial commitment is influenced by DC-derived factors such as ICOS ligand and IL-12.^{5,6} After three days, CXCR5 is up-regulated which helps the correct positioning at the T cell-B cell border facilitating cognate interactions with B cells and their subsequent entry to B cell follicles.⁷ Within the follicles, Tfh cells are characterized by constant elevated expression of CXCR5 and up-regulation of PD-1.⁸ Production of IL-21 by DCs and by the B cells at a later stage are important for the maintenance of the terminal differentiation of Tfh cells⁹ which highly express IL-21R in GCs.¹⁰ Furthermore, the interactions between Tfh cells and B cells support survival and selection signals mediated through IL-21/IL-21R and CD40/CD40L axis, and subsequent Tfh activity inducing efficient terminal differentiation of B cells.¹¹

Within the follicles, regulatory mechanisms operate to prevent the emergence of autoreactivities, while allowing the selection of high-affinity Ab-producing cells. The control of Tfh cell development and Tfh cell functions now appears to be significant for the restriction of aberrant Ab responses,^{12,13} with the possible implication of a recently described but not yet fully characterized subpopulation of T cells called follicular

regulatory T (Tfr) cells.¹⁴ Regulatory B (Breg) cells correspond to B lymphocytes that control the proportions and/or the functions of various effector cells to restrain the development of immune responses. Their depletion or defective activity skew T cell differentiation into Th1 and Th17 subsets, associated with worsened autoimmune disorders.^{15,16} Following activation through CD40 and TLR9, human B cells acquire regulatory activities suppressing cellular immune responses.¹⁷⁻¹⁹ Whether human Breg cells can affect the humoral response has never been evaluated. We elaborated an *in vitro* model for the generation of functional human Tfh cells and a co-culture system to assess the capacity of Breg cells to influence their effect on the terminal differentiation of B cells.

The presence of Breg cells alters the development and further suppress the functions of Tfh cells, repressing the GC reactions. We conclude that impaired Breg controls *in vivo* may impact the Tfh-dependent restriction of humoral responses and may lead to the development of aberrant autoimmune responses.

METHODS

T and B lymphocytes were *in vitro* differentiated into Tfh and Breg cells, respectively. To estimate the suppressive capacity of Breg cells on Tfh maturation, flow cytometry analyses and cytokine secretions were assessed on co-cultures between T and Breg cells. To determine the regulatory activity of the Bregs on Tfh function, flow cytometry, cytokine secretions and Ig productions were measured on co-cultures between unstimulated B cells, Tfh and Breg cells. To identify the influence of different cell subsets, co-cultures were performed with naïve and memory T and B lymphocytes purified by negative selection kits, and with CXCR5^{high} PD-1^{high} and CXCR5^{low} PD-1^{low} CD4⁺ T lymphocytes sorted using MoFloXDP cell sorter.

All procedures are detailed in the article's Online Repository.

RESULTS

In vitro differentiation of human follicular helper T cells.

To establish our model, T cells were polyclonally stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-12 and IL-21 (Fig E1, A), leading to the increased expression of Bcl-6^{high} IL-21^{high} CXCR5^{high} ICOS^{high} PD-1^{high} CD4⁺ T cells with up-regulation of all markers and the concomitant increased frequencies of positive T cells (Fig E2, A). This induction concerned mainly the naïve population (Fig E2, B) and was specific to the CD4⁺ T cells (Fig E3, A), resulting in a Th2 cytokine profile of $\alpha\beta$ ⁺ T cells (Fig E3, B, C). Differentiated Tfh cells were co-cultured with non-stimulated B cells (Fig E1, A). The frequencies of CD138⁺ plasma cells and IgD⁻ CD27⁺ memory B cells were up-regulated (Fig E2, C), and increased amounts of IgM, IgG and IgA were secreted (Fig E2, D) primarily by the memory B cell compartment (Fig E3, D). These experiments demonstrate that *in vitro*-generated human Tfh cells acquire efficient functional properties triggering the terminal differentiation of B cells into Ab-secreting cells.

Inhibitory effect of Breg cells on Tfh cell development.

To evaluate the role of Breg cells, B cells were stimulated through CD40 and TLR9, and these committed Bregs were added at the onset of the T cell differentiation (Fig E1, B). The induction of the Tfh markers was not observed, percentages of Bcl-6, IL-21, CXCR5, ICOS and PD-1 positive T cells remained significantly lowered, and the MFIs of the markers were not up-regulated (Fig 1, A). In the presence of non-stimulated B cells the Tfh cell maturation occurred, percentages of all markers were not affected, and the

differentiation into Bcl-6^{high} IL-21^{high} CXCR5^{high} ICOS^{high} and PD-1^{high} T cells occurred as in the absence of B cells (Fig 1, B). These findings indicate that the control of the Tfh maturation is conducted by committed Breg cells only, primarily directed to the naïve CD4⁺ T cells (Fig1, C). Although the frequency of CXCR5^{high} PD-1^{high} T cells is restrained by the Bregs differentiated from both naïve and memory B cells, Bcl-6 up-regulation is controlled by Breg-committed naïve B cells suggesting that the suppressive capacity is mainly carried by the naïve B cells (Fig E4, A, B). The inhibitory effect is also efficient on Tfh cells differentiated from splenic CD4⁺ T cells (Fig E4, C), suggesting a general immunoregulatory mechanism. When Breg cells were added on CD4⁺ differentiated Tfh cells, the expression of all markers were slightly affected, and their MFIs were not diminished (Fig 1, D), indicating that the development of fully differentiated Tfh cells can not be reversed by the Breg cells.

Mechanisms of B cell regulation on Tfh development.

Importance of cell contacts and soluble factors.

CD4⁺ T cells were stimulated in the lower chamber of Transwell systems (Fig E1, C), with Breg cells added in the upper chamber (Fig 2, A). The Breg-induced decreased percentages of Bcl-6, ICOS and PD-1 positive T cells were not observed, while the down-regulated percentages of IL-21 and to a lesser extent of CXCR5 positive T cells persisted (Fig 2, A). In the supernatant of Breg cells (Fig E1, C), the percentages of IL-21 and to a lesser extent CXCR5 positive T cells were diminished (Fig 2, B), whilst percentages of Bcl-6, ICOS and PD-1 positive T cells remained elevated (Fig 2, C).

These data indicate that the regulation of Bcl-6, ICOS and PD-1 depends on cellular contacts and that soluble factors are involved in the control of IL-21 expression, whereas modulation of CXCR5 likely requires synergistic effects of soluble factors and cell-to-cell contacts.

The molecules involved.

Beyond the previous phenotype $CD40^+ CD80^+ CD86^+ ICAM^+$,¹⁷⁻¹⁹ frequencies of $PDL-1^+$, $ICOSL^+$, $IL-21R^+$, $CD5^+$, $IL-10^+$ and $TGF\beta^+$ B cells were increased in Breg-committed cells, persisted in co-culture with T cells (Fig E5, A), and $CD5^+$ Breg cells were mostly $CD25^+$, $CD71^{+/-}$ and $CD73^-$ (Fig E5, B). To determine the molecules involved in the Breg control, blocking Abs were used in co-cultures (Fig E1, D). The expression of Bcl-6, ICOS and PD-1 was maintained (Fig 2, D) indicating the participation of CD40L, CD80, CD86 and ICAM in the Breg control. Anti-PDL-1 blocking Ab incompletely preserved the population of Bcl-6 positive T cells, suggesting that PD-1/PDL-1 interaction may be partially involved in regulating Bcl-6 expression.

The blockade of IL-10R or of $TGF\beta$ prevented the decrease in percentages of IL-21-positive T cells, whilst CXCR5 expression was preserved only after IL-10R blocking (Fig 2, E). All together, IL-10 and $TGF\beta$ are Breg-derived cytokines involved in the regulation of IL-21 and CXCR5, and of IL-21, respectively.

Suppressive activity of Breg cells on Tfh functions.

Tfh-differentiated cells and non-stimulated B cells were co-cultured for 6 days, in the presence of Breg cells (Fig E1, E). The Tfh-dependent generation of $CD138^+$ plasma

cells and IgD⁻CD27⁺ memory B cells did not occur, (Fig 3, A) and the Tfh-induced production of IgM, IgG, and IgA was inhibited (Fig 3, B). This inhibition is directed to the Ig production induced by the Tfh cells generated from naïve and from memory T cells (Fig 3, C), and is primarily directed to the terminal differentiation of the memory B cell compartment (Fig E3, D). The suppressive capacity can be supported by Breg cells originated from naïve as well as memory B cells (Fig E4, A, D).

Mechanisms of B cell regulation on Tfh functions.

Importance of cell contacts.

Tfh cells and non-stimulated B cells were co-cultured in the lower chamber of the Transwell systems for 6 days, with the Breg cells in the upper chamber (Fig E1, F). The production of IgM, IgG and IgA were recovered (Fig 3, B). These results substantiate the necessity of direct cell contacts for the Bregs to suppress the Tfh-dependent terminal differentiation of B cells into Ab-secreting cells. These effects rely on the control of IL-21R. Its induced expression on Tfh cells involved in the maintenance of their differentiation⁹, and its Tfh-dependent induction on non-stimulated B cells required for their terminal differentiation¹¹, were inhibited in the presence of Breg cells (Fig 4, A).

Induction of follicular regulatory T cells.

The percentages of Foxp3-positive T cells (Fig E1, E) within the CXCR5⁺ PD-1⁺ CD4⁺ T cells were up-regulated in the presence of Breg cells, whilst the Foxp3-positive population within the CXCR5⁻ PD-1⁻ CD4⁺ T cells was not (Fig 4, B). These data indicate

that Breg cells specifically increase the proportion of Tfr cells known to control the Tfh-dependent humoral response.²⁰

Tfh with non-stimulated B cells but also Breg cells produced TGF β , the levels of which were not increased in the co-cultures (Fig 4, C). In contrast, the levels of IL-10-Breg cells were strikingly increased. Using anti-TGF β blocking Abs, but not anti-IL-10R blocking Abs, the frequencies of Foxp3-positive T cells within the CXCR5⁺ PD-1⁺ CD4⁺ T cells were diminished (Fig 4, D) demonstrating the role for TGF β in the induction of Tfr cells. The decreased frequencies in Transwell experiments showed that cell-contacts were also necessary (Fig 4, D).

Inhibitory effect of Breg cells on in vivo purified Tfh cells.

CXCR5^{high} PD-1^{high} CD4⁺ and CXCR5⁻ PD-1⁻ CD4⁺ tonsillar T cells were sorted (Fig E6, A), polyclonally stimulated to mimic antigenic stimulation and co-cultured (Fig E6, B). CXCR5^{high} PD-1^{high} CD4⁺ Tfh cells were functional inducing higher secretions of IgM, IgG and IgA than the CXCR5⁻ PD-1⁻ CD4⁺ non-Tfh cells (Fig 5, A). In the presence of Breg cells, Ig secretions were down-regulated. Phenotypically *in vivo*-differentiated Tfh cell markers slightly decreased in the presence of Breg cells (Fig 5, B), but the percentages of positive T cells were down-regulated (Fig 5, C). Neither the phenotype nor the frequency of CXCR5⁻ PD-1⁻ CD4⁺ non-Tfh cells were affected by the addition of Breg cells (Fig 5, B, C). Finally, the frequency of *in vivo*-differentiated Foxp3-positive cells within the CXCR5^{high} PD-1^{high} sorted Tfh cell population, but not within the CXCR5⁻ PD-1⁻ sorted non-Tfh cells, increased with the Bregs (Fig 5, D). All these data

208 demonstrate that *in vivo* Tfh cells are sensitive to the suppressive activity of Breg cells,
209 and that *in vivo* Tfr cells are receptive to and their proportion increase in response to the
210 Breg signals.

211

DISCUSSION

We characterize cooperative functions for IL-12 and IL-21 to generate human Tfh cells *in vitro*. These differentiated Tfh cells were functional helping human B cells to differentiate into plasma and memory B cells allowing the secretion of Igs. Our study supplements the great diversity of signals that produce efficient Tfh cells.²¹⁻²⁸

Moreover, we show for the first time that human Breg cells can develop negative effects on humoral immune responses towards the control of the generation and the functions of Tfh cells. Stimulation with CD40 and TLR9 was prerequisite, as non-stimulated B cells that may contained *in vivo* pre-conditioned Bregs, were unable to affect the Tfh cells in co-cultures. The committed Breg cells, primarily originated from naïve B cells, down-regulated the Tfh specific markers on tonsillar and splenic differentiated T cells, mainly originated from naïve CD4⁺ T cells. A decreased frequency using *in vivo* sorted Tfh cells was also observed. In the follicles, the maturation into Tfh cells occurs in the T cell zone and at the T cell:B cell border of the GCs. Hence, Breg cells have the capacity to inhibit the Tfh development within secondary lymphoid tissues. CD40+TLR9 stimulation up-regulated the CXCR5 expression on Breg surface (Fig E5, C), suggesting that the Breg cells can also migrate within the GCs to control the Tfh functions. Thus, Bregs, derived from naïve or memory B cells, inhibited the terminal differentiation of B cells and the associated secretion of Igs induced by *in vitro*- as well as *in vivo*- differentiated Tfh cells, originated either from naïve or from memory CD4⁺ T cells. The recent description of the CD25⁺ Bcl6^{low} CXCR5⁺PD-1⁺ CD4⁺ Tfh cells that

interact with the B cells to support their terminal differentiation in the GC of human tonsils,²⁹ suggests that the suppressive effect of the Bregs might be preferentially directed to this Tfh subpopulation. We have previously shown that human Breg cells inflect the growth of the cellular immune response.^{17,19} Our current study further demonstrates the ability of human Breg cells to modulate humoral immune responses through the control of the maturation and the functions of Tfh cells.

Two groups of B cells have been identified with different regulatory potential in murine models.^{30,31} In human, CD24^{hi} CD38^{hi} CD5⁺ B cells,^{17,18} CD24^{hi} CD27⁺ B cells,³² but also CD25⁺ CD71⁺ CD73⁻ B cells,³³ were associated with suppressive effects. In our study, Bregs are CD19^{hi} IgD⁺ CD27⁻ CD38^{hi} CD24^{hi} CD40^{hi} CD80^{hi} CD86^{hi} CD5⁺ CD25⁺ CD71^{+/-} CD73⁻ PDL-1⁺ ICAM-1⁺ ICOS-L⁺ and IL-21R⁺. This alternative phenotype suggests a plasticity of the Breg cells to maintain appropriate contacts with the target cells. The intercellular contacts with T cells,¹⁷ DCs,¹⁹ or Tfh cells likely alter the expression of cell surface molecules on the Breg population to settle adapted suppressive capacities.

IL-10 and TGF β play an important role in the inhibitory function of Breg cells.³¹⁻³⁶ Our experiments demonstrate that they are also essential to modulate the IL-21 and CXCR5 expressions on Tfh cells. Tfr cells that are distinct Treg cells located in GCs,^{14,20,37,38} are identified as CXCR5^{hi} PD-1^{hi} Foxp3⁺ CD4⁺ T cells.³⁸ In mice, they exert an IL-10- and TGF β -dependent inhibitory effect on GC-located Tfh cells and on the humoral B cell response. Our study suggests a Breg-dependent increase of the Tfr cell

proportion that likely participate to the restriction of the Tfh terminal differentiation and functions reducing the humoral response in humans, with a preponderant role ascribed to TGF β . Transwell experiments indicate also the requirement of cellular interaction, which is consistent with the role assigned to TGF β and cell surface CTLA-4 in the regulation of the humoral response.³⁹ However, it is still unclear how these cells operate. Tfr cells could directly act on B cells⁴⁰ while Breg cells could also directly modulate Tfh cell functions. These options remain to be explored.

Finally, we observed the induction of IL-21R on Tfh cells and its Tfh-mediated induction on B cells as expected.^{9,10,41} The Breg-mediated reduction arises as an additional negative feedback to regulate the Tfh-dependent GC responses. In this context, the maturation of B cells secreting IL-10 requires IL-21 and CD40.^{42,43} The high density level of IL-21R observed on the Breg cells might explain their prominent sensitivity to regulate the Tfh cells and the associated humoral responses.

In conclusion, it becomes clear that the proportion of Tfh cells relative to the neighbouring Tfr and Breg cells will influence the balance modulating the humoral responses and tolerance achievement. It is noteworthy that abnormal functions of Tfh cells cause deleterious effects and trigger autoimmunity.⁴⁴⁻⁴⁹ A better understanding of human Tfh, Tfr and Breg inter-relations is worthy of pursuit to elaborate new therapeutic strategies specifically in autoimmune disorders in which suppressive Breg functions are lacking.

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- 424

FIGURES LEGENDS

FIG 1. Breg cells inhibit Tfh cell development but not differentiated T cells.

Expression of Tfh markers on CD4-positive stimulated T cells co-cultured with Breg cells (**A**) or non-stimulated B cells (**B**). **C**, Percentage of cells from purified CD4⁺ T cells. **D**, Expression of all markers on already differentiated Tfh cells co-cultured with Breg cells **P*<0.05; ***P*<0.01 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG 2. Characterization of Breg suppressive activity on Tfh development.

Expression and percentages at day 5 (**A**) of Tfh markers on stimulated T cells co-cultured in a Transwell (TW) system. Time course (**B**) and percentages (**C**) with Breg cells or their supernatant (spt). Percentages (**D**, **E**) after co-culture with blocking antibodies. **P*<0.05; ***P*<0.01; ****P*<0.001 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG 3. Breg cells suppress Tfh cell function.

Frequencies of CD138⁺CD19⁺ plasma cells and CD19⁺IgD⁻CD27⁺ memory B cells during co-cultures of B and Tfh cells with Breg cells (**A**). Concentration of Igs in the supernatants (**B**) and after co-cultures with Tfh cells from purified naïve and memory T cells (**C**). TW: Transwell system **P*<0.05; ***P*<0.01 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG 4. Characterization of Breg suppressive effects on Tfh cell function.

A, IL-21R expression on T cells and on B cells. **B**, Percentages of Foxp3-positive T cells. **C**, Secretion of TGFβ and IL-10. **D**, Percentages of Foxp3-positive T cells within the CXCR5⁺PD-1⁺ T cells after co-culture with blocking antibodies or in Transwell (TW) system. **P*<0.05; ***P*<0.01; ****P*<0.001 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG 5. Breg cells suppress the function of *in vivo* differentiated T cells. A,
Concentration of Igs after co-cultures of B cells with *in vivo*-differentiated
CXCR5^{high}PD-1^{high} Tfh cells or CXCR5-PD-1- T cells and Breg cells. **B,**
C, Expression of Tfh markers on sorted T cells after co-cultures. **D,**
Percentages of Foxp3-positive T cells within the sorted T cells after co-
cultures. * $P < 0.05$; ** $P < 0.01$ (Wilcoxon and Mann-Whitney tests).
Mean $\pm$ SEM of 6 experiments.

Figure 1

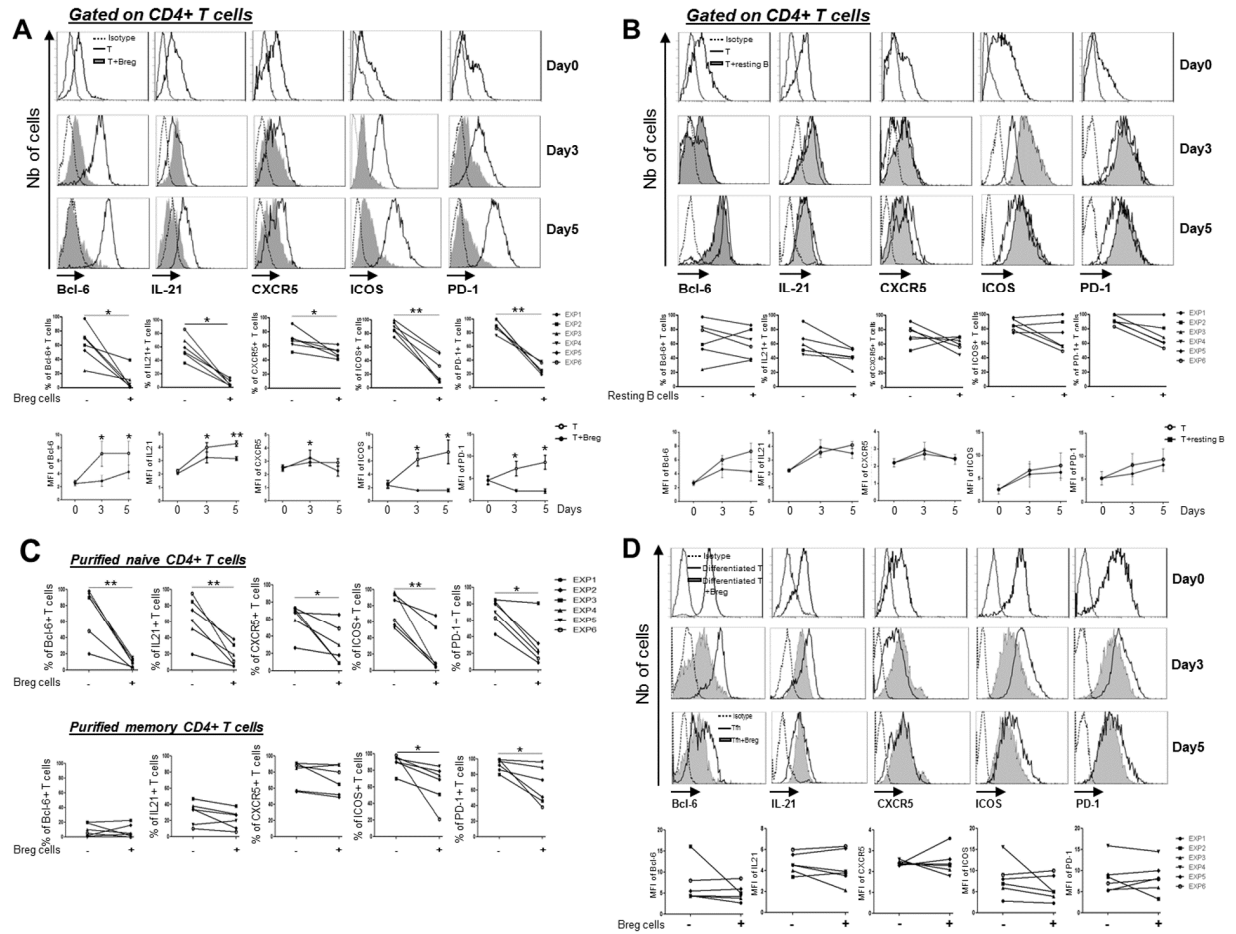


Figure 2

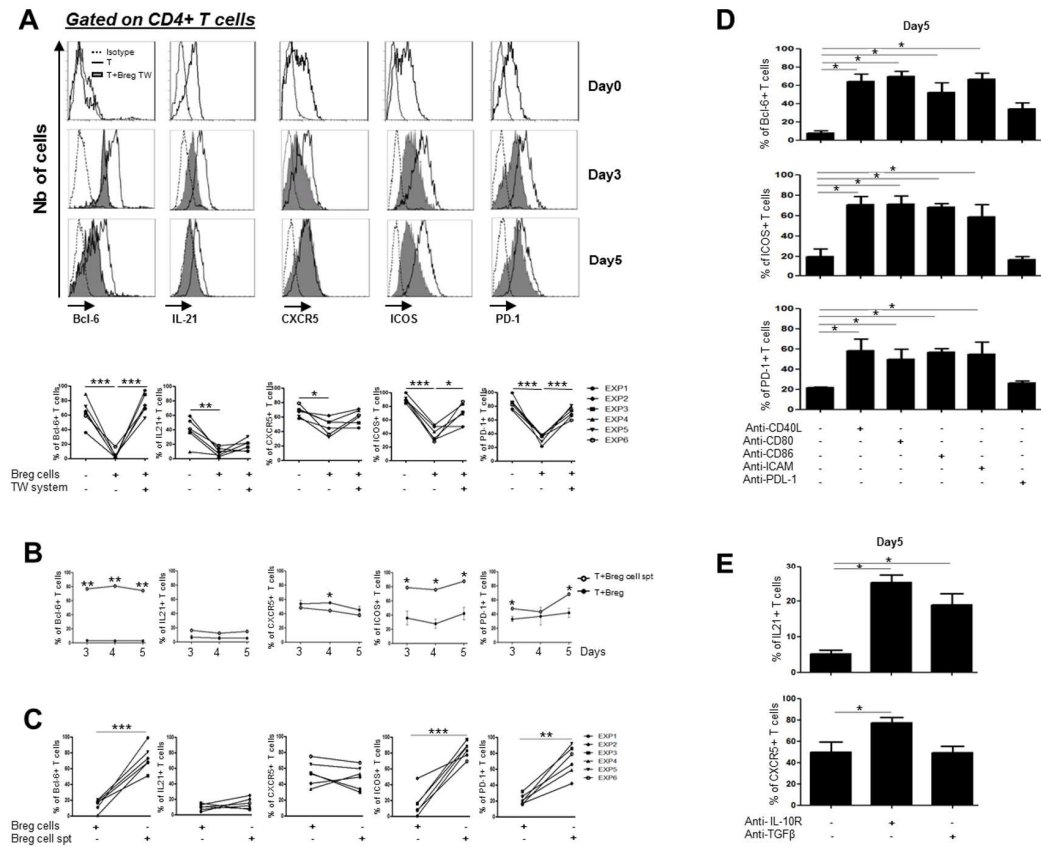


Figure 3

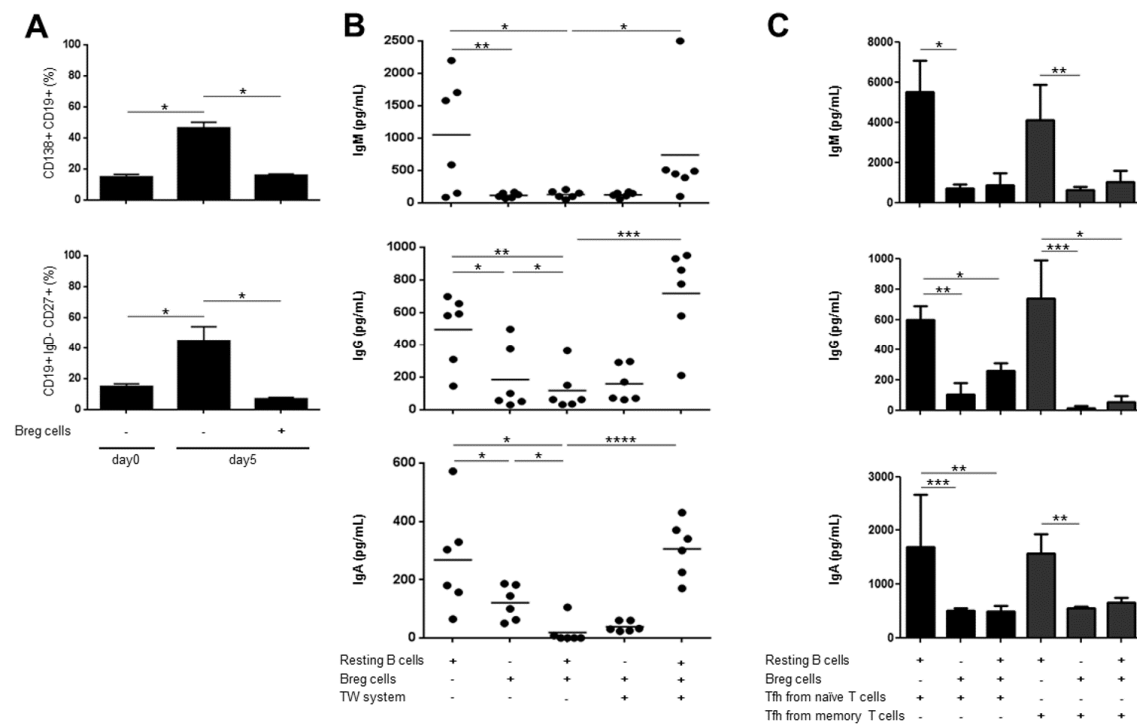


Figure 4

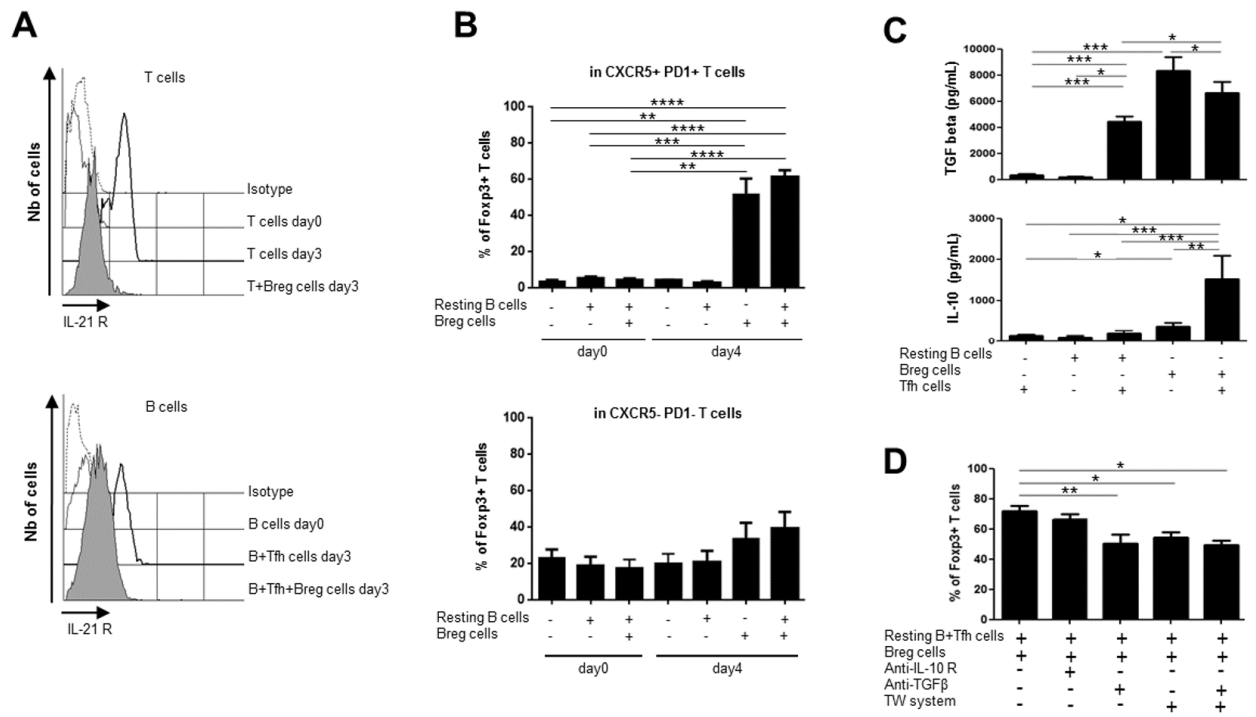
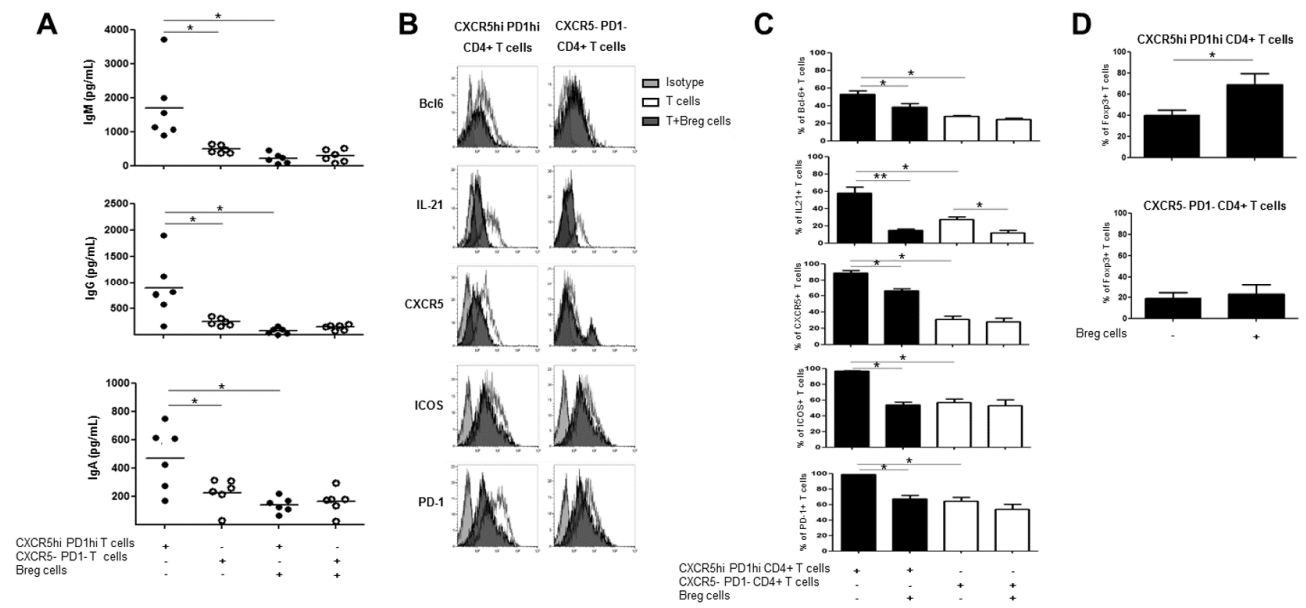


Figure 5



METHODS

SUPPLEMENTARY DATA

Isolation of cells. T and B lymphocytes were purified from children tonsils after routine tonsillectomy and from splenic tissue after splenectomy from healthy individual. Ethical approval was provided by the Institutional Review Board of the Brest University Hospital. Cell suspensions were isolated by Ficoll-Hypaque centrifugation, and T cells and B cells purified by negative selection with T cell and B cell enrichment kits (StemCell Technologies) according to the manufacturer's instructions. Preparations in all experiments were >98% pure, and in the figures, relate to different donors.

In some experiments, naïve and memory B and CD4⁺ T cells were purified by negative selection kits (Stem Cell Technologies), and CXCR5^{high} PD-1^{high} and CXCR5⁻ PD-1⁻ CD4⁺ T cells were sorted using a MoFlo XDP flow cytometer (Beckman Coulter).

Stimulation of T and B cells. T cells were seeded at 2×10^5 cell/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine (Invitrogen Life Technologies), 200 U/ml penicillin and 100 µg/ml streptomycin (RPMI-1640 complete medium). They were polyclonally stimulated with 1µg/ml anti-CD3 and anti-CD28 mAbs¹ on anti-mouse IgG-Fc coated 96-well plates in the presence of 10ng/ml IL-12 and IL-21 (ImmunoTools) for up to 5 days.

B cells were seeded at 5×10^5 cell/ml on 5×10^5 mitomycin C-treated NIH-3T3 fibroblasts transfected with human *CD40L* gene and in the presence of 0.25µM CpG-ODN 2006 (Caylo-InvivoGen). They were stimulated for 3 days in 24-well plates in RPMI-1640 complete medium to induce their differentiation into Breg cells.^{2,3}

Co-culture assays. Breg cells were added to T cells at the onset of their activation at 1:1 ratio, and the Tfh cell development analyzed by flow cytometry. For the blocking

experiments, 10µg/ml anti-CD40L (R&D Systems), anti-PDL1 (eBioscience), anti-CD80 (ImmunoTools), anti-CD86 (ImmunoTools), anti-ICAM, anti-IL-10Rα and anti-IL-10Rβ or anti-TGFβ (R&D Systems) Abs were added at the onset of the co-cultures.

1x10⁵ Tfh cells were added to non-stimulated B cells at a 1:1 ratio for 6 days in RPMI-1640 complete medium. The terminal differentiation of B cells into Ab-producing cells and memory B cells was evaluated by flow cytometry and ELISA. Breg cells were added at a 1:1:1 ratio. For the experiments in Transwells, Tfh and non-stimulated B cells were co-cultured in the lower chamber, and Breg cells added in the upper chamber.

Flow cytometry. Cells were analyzed at the onset (Day 0) and up to 5 or 6 days of cultures. All conjugated mAbs were purchased from Beckman Coulter unless otherwise indicated. We used FITC-anti-CXCR5 (BD Biosciences), PE-anti-ICOS, ECD-, PE-Cy7-or APC-anti-CD19, PE-Cy5- or PE-Cy7-anti-CD4, and PE-Cy7-anti-PD-1; FITC-anti-CD71, CD80, ICAM, IgD (BD Biosciences), FITC- or PE-Cy7-anti-CD5, PE-anti-CD27, CD40, CD73 (BD Biosciences), CD86, CD138, PDL-1, ICOSL (BD Biosciences), IL21R (BD Biosciences), PE-Cy5.5-anti-CD25 mAbs. For intracellular stainings, cells were permeabilized using the cytofix/cytoperm permeabilization kit (BD Biosciences) and incubated with FITC-anti-Bcl-6 (Abcam), IL-10, TGFβ (R&D Systems), PE-anti-IL-21 or Foxp3 (BD Biosciences) Abs according to the manufacturer's instruction.

ELISA. ELISA microtiter plates were coated overnight with 10µg/ml anti-IgM, anti-IgA (Jackson ImmunoResearch) or anti-IgG (Dakopatts) Abs and blocked with 3% BSA for 2 hours at 37°C. After 3 washes in PBS, 100µl of 1/100 diluted supernatants was added and incubated for 1 hour at 37°C. Incubation with horseradish peroxidase-conjugated anti-IgM, -IgG or -IgA Abs (Dakopatts) was then performed for a further 1 hour at room temperature, followed by incubation with OPD as substrate. After addition of sulfuric acid to stop the

colorimetric reaction, absorbance was measured at 492nm. PBS and RPMI-1640 complete medium were used as negative controls.

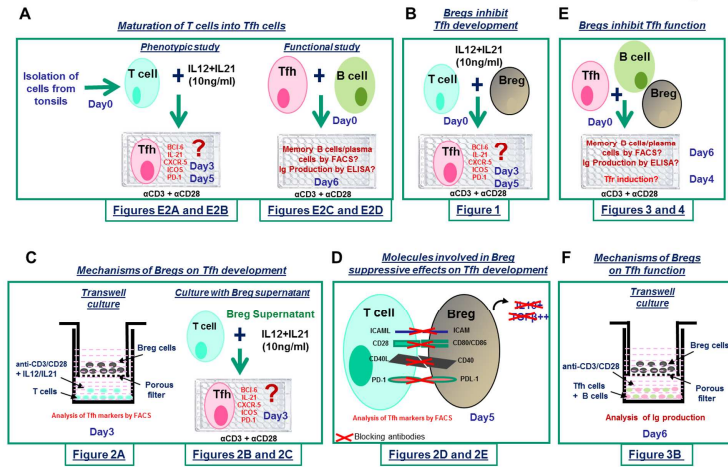
IL-10 and TGF β in the supernatants of cultured and co-cultured cells were measured with commercial kits from Beckman Coulter and R&D Systems, respectively. RPMI1640 complete medium was used as negative control.

Statistical analysis. Data were expressed as mean $\pm$ SEM. Statistical analyses were performed with Prism4 Graphpad Software. Comparisons were performed with the Wilcoxon test and Mann-Whitney test for paired and unpaired data, respectively. $p < 0.05$ was considered as significant.

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Figure E1



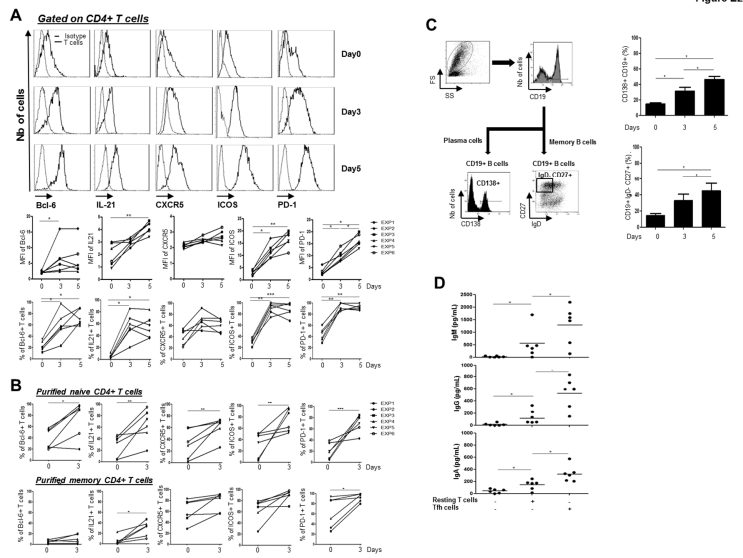


Figure E3

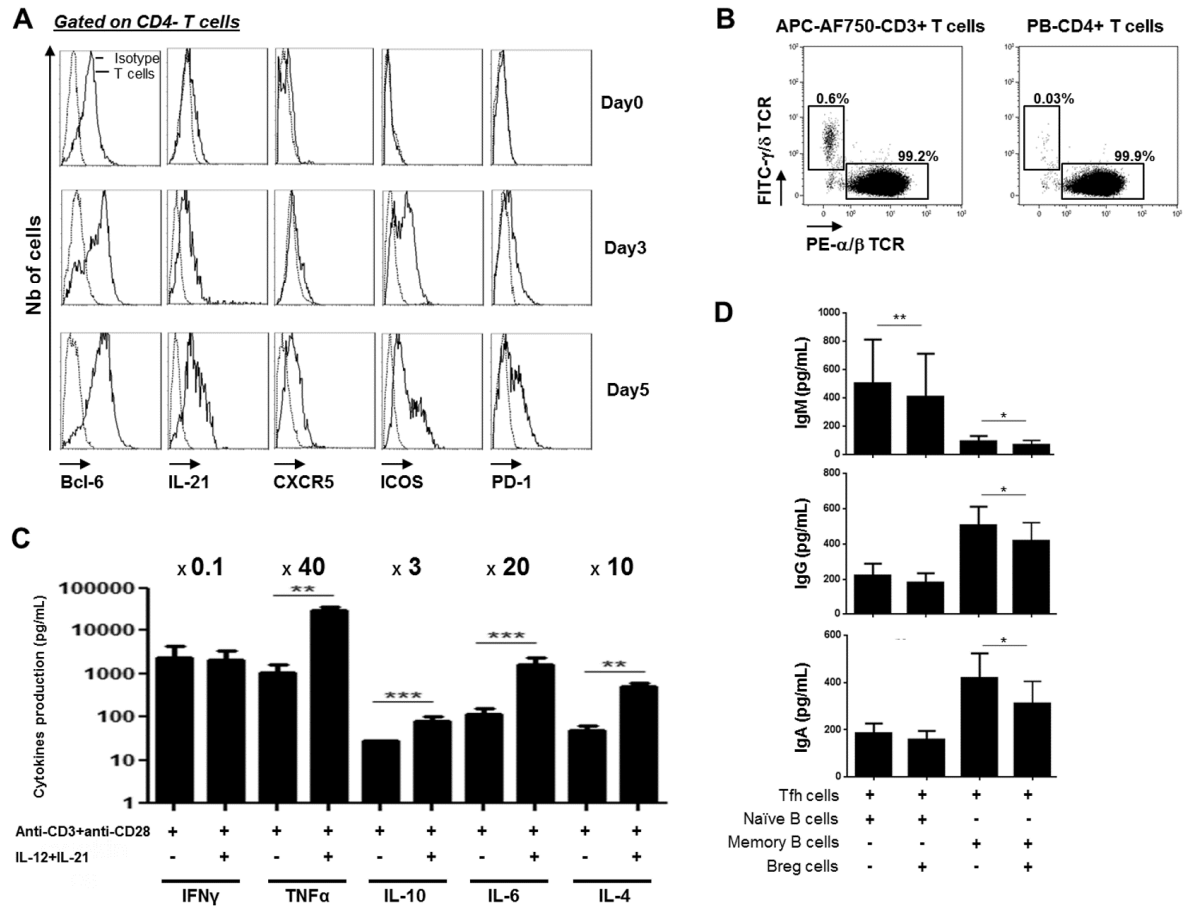


Figure E4

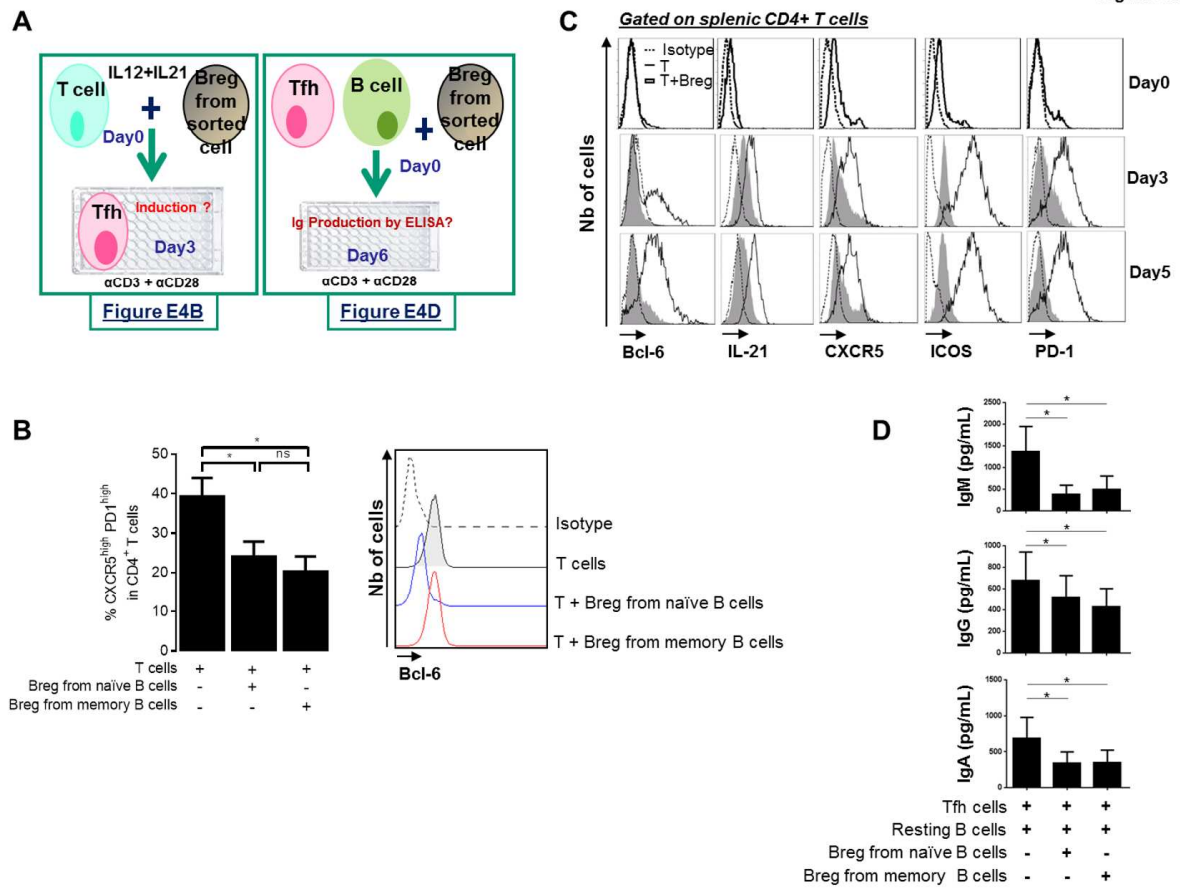


Figure E5

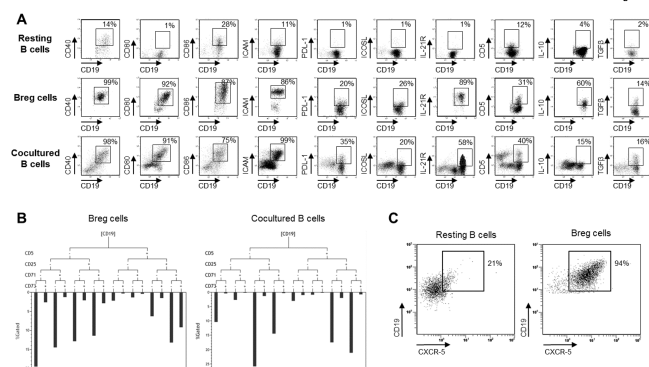
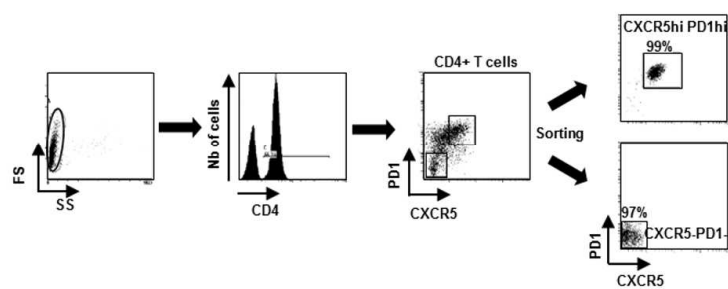


Figure E6

A



B

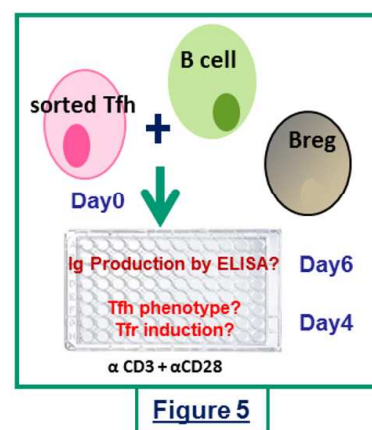


Figure 5

FIGURES LEGENDS

SUPPLEMENTARY DATA

FIG E1. Experimental schemes. Description of experiments from the Figure E2 (A), Figure 1 (B), Figure 2 (C, D), Figure 3 (E, F) and Figure 4 (E).

FIG E2. Maturation of T cells into functional follicular helper T cells. A, Representative example of the expression of Tfh markers on CD4-positive stimulated T cells, MFI and percentages of CD4-positive T cells. B, Percentage of positive T cells from purified naïve and memory CD4⁺ T cells. C, Gating strategy and Percentages of CD138⁺CD19⁺ plasma cells and CD19⁺IgD⁺CD27⁺ memory B cells after co-culture of B cells with Tfh-differentiated T cells. D, Concentration of IgM, IgG and IgA secreted in the supernatants of co-cultures *P<0.05; **P<0.01; ***P<0.001 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG E3. A, CD4-negative T cells do not differentiate into follicular helper T cells. Representative example of the expression of Tfh markers on CD4-negative T cells polyclonally stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-12 and IL-21. B, γ/δ TCR and α/β TCR expression on tonsillar T cells. Cells were stained at day 0 with FITC-anti- γ/δ TCR, PE-anti- α/β TCR, APC-AF750-anti-CD3 and PB-anti-CD4 and analyzed on Navios Flow cytometer. C, Cytokine profile of *in vitro* differentiated follicular helper T cells. Concentration of Th1 (IFN γ , TNF α) and Th2 (IL-10, IL-6, IL-4) cytokines in the supernatant of tonsillar T cells stimulated for 5 days with anti-CD3 and anti-CD28 Abs with or without IL-12 and IL-21 measured using commercial kits (Beckman Coulter). D, Tfh cells require the presence of memory B cells to acquire efficient functional properties. Tonsillar CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-12 and IL-21 for 3 days. These differentiated Tfh cells were co-cultured for 6 days with purified naïve or memory B cells in the presence of Breg cells. Concentration of IgM, IgG and IgA secreted in the supernatants. *P<0.05; **P<0.01; ***P<0.001 (Wilcoxon test). Mean±SEM of 6 experiments.

FIG E4. Efficiency of Breg cells. A, Experimental scheme of co-culture experiments of tonsillar T cells with sorted naïve B cells or memory B cells committed into Breg

cells. **B**, The differentiation of tonsillar CD4-positive T cells into CXCR5^{high} PD1^{high} Bcl-6^{high} T cells is mainly inhibited by the naïve B cells committed into Breg cells. **C**, The differentiation of splenic CD4-positive T cells into Tfh cells is inhibited by splenic B cells committed into Bregs. Representative example of the expression of Tfh markers on CD4-positive T cells isolated from spleen and stimulated with anti-CD3 and anti-CD28 Abs in the presence of IL-12 and IL-21 with or without Breg cells. **D**, The function of Tfh cells is inhibited by naïve as well as memory B cells committed into Breg cells. Concentration of IgM, IgG and IgA in the supernatants after co-cultures with Breg cells from sorted naïve and memory B cells. *P<0.05 (Mann-Whitney test). Mean±SEM of 6 experiments.

FIG E5. Phenotype of CD19-positive B cells. **A**, Phenotypic analysis of B cells before stimulation, after TLR9+CD40 stimulation for their differentiation into Breg, and after co-culture with T cells. **B**, Frequencies of CD5, CD25, CD71 and CD73 expression on the CD19⁺ B cells after TLR9+CD40 stimulation (Breg cells) and after co-culture (Cocultured B cells). **C**, Expression of CXCR5 on B cells stained with FITC-anti-CXCR5 and ECD-anti-CD19 Abs before (Resting B cells) and after 3 days of TLR9+CD40 stimulation (Breg cells).

FIG E6. Breg cells suppress the function of *in vivo* differentiated T cells. **A**, Strategy for the sorting of *in vivo* differentiated CXCR5^{hi}PD-1^{hi} and CXCR5-PD-1⁻ CD4⁺ positive T cells. **B**, Experimental scheme of co-culture experiments (Figure 5) of sorted T cells and B cells with or without Breg cells.