

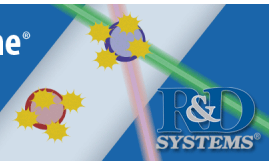


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CUTTING EDGE

Cutting Edge: Direct Suppression of B Cells by CD4⁺CD25⁺ Regulatory T Cells¹Hyung W. Lim,* Peter Hillsamer,[†] Allison H. Banham,[‡] and Chang H. Kim^{2*}

Regulatory T cells (Tregs) can potentially migrate to the B cell areas of secondary lymphoid tissues and suppress T cell-dependent B cell Ig response. T cell-dependent Ig response requires B cell stimulation by Th cells. It has been unknown whether Tregs can directly suppress B cells or whether they must suppress Th cells to suppress B cell response. We report here that Foxp3⁺ Tregs are found in T-B area borders and within germinal centers of human lymphoid tissues and can directly suppress B cell Ig response. Although Tregs can effectively suppress T cells, they can also directly suppress B cell response without the need to first suppress Th cells. The direct suppression of B cell Ig production by Tregs is accompanied by inhibition of Ig class switch recombination. The Journal of Immunology, 2005, 175: 4180–4183.

Thelper cells in secondary lymphoid tissues are thought to play important roles in providing cytokine and costimulation signals to B cells for Ig synthesis (1–4). Upon stimulation by Ags and T cells in the T-B area borders adjacent to follicles, B cells become short-lived Ab-forming cells and some of these cells enter follicles for germinal center (GC)³ response (5). GCs are dedicated follicles for B cell expansion, somatic hypermutation, and class switch recombination (CSR), processes that are regulated by T cells, follicular dendritic cells, and other cells (6). Regulatory T cells (Tregs) appear to play important roles in regulation of B cell Ig response. In several autoimmune diseases with aberrant Ab production, the function or number of Tregs is decreased (7–11). Depletion of Tregs can lead to aberrant Ab production (10, 12). Furthermore, administration of Tregs into autoimmune animals significantly reduced autoantibody response (13). We previously found that Tregs can up-regulate the B cell zone homing receptor CXCR5 upon Ag priming and can suppress T cell-dependent B cell Ig production (14).

It has been unknown whether Foxp3⁺ Tregs can directly suppress B cells to suppress Ig production or whether they have to suppress Th cells to indirectly suppress the B cell response. In

this report, we provide evidence that Foxp3⁺ Tregs are present in B cell areas where T-B cell interaction and humoral immune responses are believed to occur, and that they can directly suppress B cell Ig production and CSR without having to suppress Th cells.

Materials and Methods

Immunohistochemistry and flow cytometry

Frozen sections of tonsils were acetone-fixed and stained by polyclonal goat anti-Foxp3 (Novus Biologicals) or monoclonal anti-hFoxp3 mIgG1 Ab (236A/E7; Banham laboratory or Abcam) (15). Anti-goat/mouse-biotin and the VECTASTAIN ABC kit (Vector Laboratories) were used to assess the expression of Foxp3. Frozen sections were separately stained by anti-IgD-FITC or with anti-CD4-FITC (Caltag Laboratories) and then with anti-FITC-AP to visualize follicles and T cell areas, respectively. For confocal microscopy, the frozen tonsil sections were stained with anti-IgD, anti-CD3, and anti-Foxp3 as described previously (16). For flow cytometry analysis, tonsil CD4⁺ T cells were first stained with surface Abs (anti-CD4, anti-CD25, and anti-CD69), fixed, permeabilized, and then stained with anti-hFoxp3 (PCH101; eBioscience).

Cell isolation, cell culture, and ELISA

CD57[−]CD69[−]CD25⁺CD4⁺ Tregs (purity >95%) were isolated by multi-step magnetic sorting from human tonsil mononuclear cells as described previously (14). Naive CD19⁺IgD⁺CD38[−] tonsil B cells (purity >99%) and CD19⁺CD38⁺ GC-B cells (purity >95%) were isolated as described previously (17). Naive B cells were activated for 2 h at 4°C with Sepharose-conjugated rabbit Ab to human Ig μ -chain and rabbit Ab to human Ig (H + L) chain (Irvine Scientific; mixed 1:1 at 2 μ g/ml). Indicated numbers of Tregs were cocultured with B cells in the presence of staphylococcal enterotoxin B (SEB; 1 μ g/ml; Sigma-Aldrich) in a 5% CO₂ incubator at 37°C for 5 days. For T cell-free B cell stimulation, anti-CD40 (5 μ g/ml; BD Pharmingen) and cytokines (0.25 ng/ml IL-2, 20 ng/ml IL-4, 40 ng/ml IL-10; R&D Systems) were used. Concentrations of Igs and CXCL13 in the supernatants were determined by ELISA as previously described (14, 16). When indicated, neutralizing Abs were added at following concentrations: anti-TGF- β 1 (R&D Systems; clone 9016.2, 10 μ g/ml), anti-CTLA-4 (BD Pharmingen; clone BNI3, 10 μ g/ml), and IgG1 isotype Ab (R&D Systems; clone 11711.11, 10 μ g/ml).

Analyses of activation-induced cytosine deaminase (AID), Ig-productive and circle transcripts, and switch circles

AID, Ig transcripts, and β -actin were amplified by PCR as previously described (17). Circle transcript analysis was performed using specific primer sets designed to detect only circle transcripts but not productive or germline transcripts (18). Digestion circularization (DC)-PCR was performed as previously described using PCR primers for γ 3 and α 1/2 (19).

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³ Abbreviations used in this paper: GC, germinal center; CSR, class switch recombination; Treg, regulatory T cell; SEB, staphylococcal enterotoxin B; AID, activation-induced cytosine deaminase; DC-PCR, digestion circularization-PCR; PHA, phytohemagglutinin.

Results and Discussion

Foxp3⁺ Tregs are found in B cell-rich areas

Foxp3 is a transcriptional repressor (20) mutated in scurfy mice that lack conventional CD4⁺CD25⁺ Tregs (21) and in human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome patients with autoimmune diseases (22). Foxp3 is specifically expressed by T cells with regulatory functions (23) and therefore is an appropriate Ag marker for Tregs. To determine the distribution of Foxp3⁺ cells in T-zone and B cell areas, we performed immunohistochemistry on human tonsil sections using anti-Foxp3 Abs (Fig. 1A). We also separately stained serial tonsil sections with anti-IgD or anti-CD4 to identify T-zone, B follicle, and T-B border areas. The majority of Foxp3⁺ T cells were localized in the T cell zone. It was notable that quite a few Foxp3⁺ cells were found in T-B border areas including mantle zones (a cell layer enriched with IgD⁺ B lymphocytes surrounding the GCs of lymphoid follicles). Clearly, small but significant numbers of Foxp3⁺ T cells were seen within the GCs. The presence of Foxp3⁺ T cells in the T-B border areas was interesting, and we used a three-color confocal microscopy technique to visualize the presence of Foxp3⁺ cells along with IgD⁺ B cells and T cells in the T-B border areas. All Foxp3⁺ cells were T cells (CD3⁺), and some Foxp3⁺ T cells were in contact with T as well as IgD⁺ B cells. When normalized for CD3⁺ T cell numbers, the frequency of Foxp3⁺ T cells in T-B border areas exceeded that of T cell or GC areas (Fig. 1B).

Almost all Foxp3⁺ cells in human tonsil were CD4⁺ but not CD8⁺ T cells (Fig. 1C). The Foxp3⁺CD4⁺ T cells were divided into CD45RA[−] (memory type) and CD45RA⁺ (naive type) cells. The CD4⁺CD25⁺ T cells in tonsils can be divided into resting CD4⁺CD25⁺CD69[−] and activated CD4⁺CD25⁺CD69⁺ T cell fractions based on CD69 expression. The CD4⁺CD25⁺CD69[−] cell fraction was highly enriched with Foxp3⁺ T cells (Fig. 1D). In contrast, only 40–50% of the CD4⁺CD25⁺CD69⁺ cells were Foxp3⁺ cells, showing that this population contains many non-Tregs such as activated conventional T cells. We used this CD4⁺CD25⁺CD69[−] T cell population enriched with Foxp3⁺ cells in the suppression study described in Fig. 2.

Tregs can directly suppress B cell Ig response without having to suppress Th cells

A human CD57⁺ GC-Th cell subset is specifically present within GCs, and they are highly efficient T helpers in stimulating B cells for production of Igs (3, 17). Upon Ag priming, certain Tregs with the CD4⁺CD25⁺CD69[−] phenotype up-regulate the B cell area homing chemokine receptor CXCR5 and can suppress GC-Th cell-stimulated B cell Ig production (14). However, it is unknown whether these Tregs can suppress Th cells to suppress the B cell response and/or whether they can directly act on B cells to suppress the B cell response. To address this issue, we determined the suppressive capacity of Tregs on T vs B cells separately from each other (Fig. 2, A–C). The following two indicators were used to assess the T vs B cell responses: CXCL13 production (a GC-Th cell response) (16, 24) and production of IgG and IgA (B cell responses). Tregs were able to suppress the B cell response induced in a T cell-free activation condition (anti-CD40, IL-2, IL-4, and IL-10) (Fig. 2B). Tregs were also able to suppress the T cell response (GC-Th cell production of CXCL13) induced by phytohemagglutinin (PHA) in a B cell-free activation condition (Fig. 2C). These results sug-

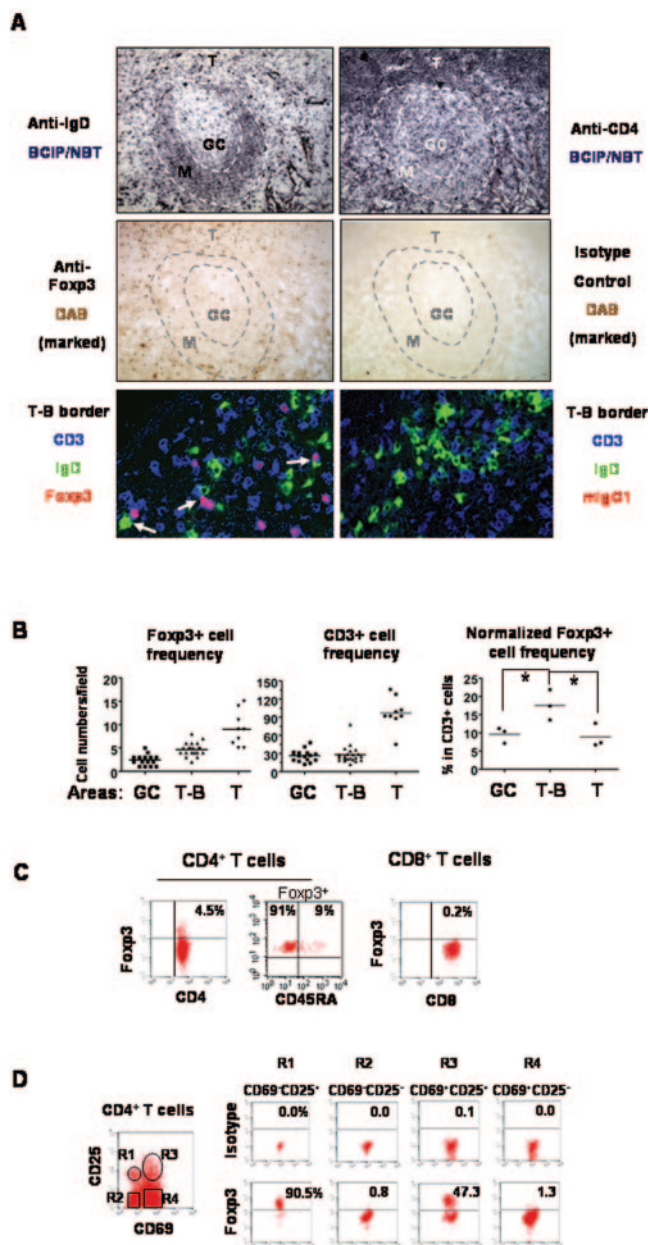


FIGURE 1. A, Foxp3⁺ cells are present in follicles and T-B borders. Serial frozen sections of human tonsils were separately stained for IgD, CD4, and Foxp3. Many Foxp3⁺ cells are in T cell and T-B border areas including the mantle zone (M). Some Foxp3⁺ cells are within GCs. The Foxp3⁺ cells in T-B border areas were visualized with confocal analysis, where some Foxp3⁺ cells (red nucleus with blue cytoplasm indicated by arrows) were in contact with CD3⁺ T cells (blue) and IgD⁺ B cells (green). Representative data from three independent experiments using different specimens are shown. B, The frequencies of Foxp3⁺ cells and CD3⁺ T cells (cell numbers per field; each field = 0.0145 mm²) are shown along with normalized Foxp3⁺ T cell frequencies (percentage of CD3⁺ cells). The counts were from many different fields and images obtained from three different specimens. *, Significant difference (*p* < 0.05). C and D, Foxp3⁺ T cells are enriched in the CD4⁺CD25⁺CD69[−] T cell population. Tonsil mononuclear cells were examined by FACS after staining with anti-Foxp3-PE and indicated Abs to surface Ags.

gest that Tregs can directly suppress B cells in addition to T cells.

Generally, Tregs need cell-cell contact with target cells to suppress them. When the Tregs were separated from B cells, B cell response was not suppressed (Fig. 2D), suggesting that the

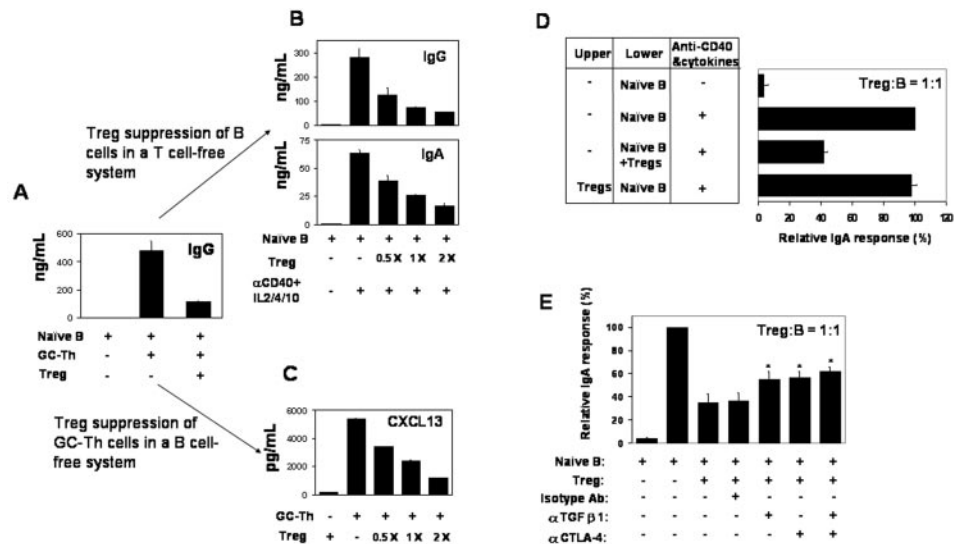


FIGURE 2. A–C, Tregs can directly suppress B cells. B cells were stimulated by CD57⁺ GC-Th cells and SEB (A) or anti-CD40 + IL-2 + IL-4 + IL-10 (B). CD57⁺ GC-Th cells were stimulated by PHA (C). For B, naive B cells were cultured in 96-well (1×10^5 /well) plates in the presence of anti-CD40 and cytokines (IL-2, IL-4, and IL-10) for 5 days. CD4⁺CD25⁺CD69⁻ Tregs were added at indicated ratios to the cultures of B cells and/or T cells. SEB was added to culture to activate Tregs. Treg suppression of IgG and IgA (B cell responses) and CXCL13 (a T cell response) secretion was assessed. D, Tregs suppress B cells through cell-cell interaction. Transwells with 0.4 μ m pores were used to separate the Tregs and B cells (ratio = 1:1). PHA was added to all cultures except the first culture (control) to stimulate Tregs. E, The direct B cell suppression by Tregs is only partly mediated by CTLA4 and TGF- β . IgA production is shown for D and E, but an almost identical pattern of suppression was observed with IgM and IgE as well (data not shown). IgG was not examined because the neutralizing Abs cross-react with the IgG capturing Ab. Representative data (A, B, and C) or combined data (D and E) from three independent experiments are shown.

direct B cell suppression activity of Tregs requires cell-cell contact between B cells and Tregs. Although controversial, TGF- β 1 and CTLA4 are implicated in Treg suppression of non-Tregs. We used neutralizing Abs to block these molecules in culture. Abs to TGF- β 1 and CTLA4 had partial (~30%) blocking effects (Fig. 2E), suggesting possible involvement of these molecules as well as other unidentified molecules in Treg suppression of B cells.

Tregs can directly suppress B cell CSR

We then determined whether Tregs can suppress AID expression, expression of productive Ig transcripts and circle transcripts, and appearance of switch circles to assess their ability to regulate Ig class switch in a T cell-free activation condition (Fig. 3). Tregs were able to suppress the expression of AID, a key enzyme involved in CSR and affinity maturation, in a T cell-free activation condition (Fig. 3A). Tregs suppressed the appearance of γ 3 and α 1/2 switch circles (Fig. 3B), by-products of IgG3 and IgA CSR, respectively, determined by a DC-PCR method. Tregs suppressed the expression of productive transcripts for IgG1–4 and IgA1–2 (Fig. 3C) and I γ -C μ and I α -C μ circle transcripts induced by anti-CD40, IL-2, IL-4, and IL-10 (Fig. 3D). We also investigated whether Tregs can suppress the above-mentioned B cell Ig production events when B cells are stimulated by GC-Th cells. Tregs suppressed generation of switch circles, expression of productive transcripts, and expression of circular transcripts induced by GC-Th cells (Fig. 4). These results suggest that B cell Ig CSR is severely suppressed in the presence of Tregs.

Tregs have the capacity to directly regulate B cell Ig production

Through the study, we addressed several important issues regarding Treg suppression of B cell responses. First, our results show that Foxp3⁺ T cells exist in the T-B area borders and

within GCs, the areas where B cells interact with Th cells and undergo Ig production processes. This data is consistent with the fact that CD4⁺CD25⁺CD69⁻ Tregs, upon TCR activation, rapidly up-regulate the B cell area homing receptor

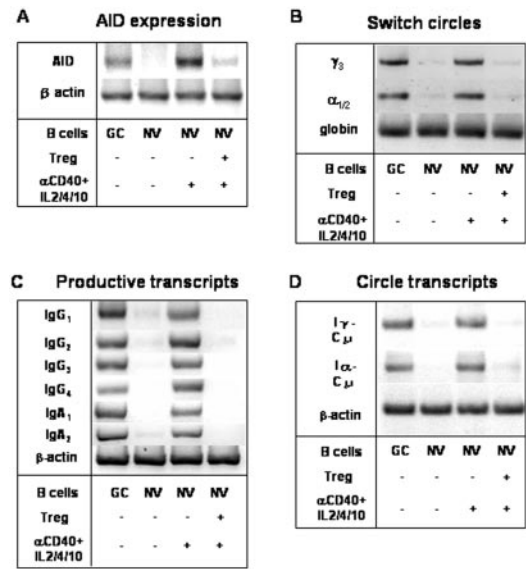


FIGURE 3. Tregs can suppress B cell expression of AID (A), generation of Ig switch circles (B), and expression of Ig productive transcripts (C) and circle transcripts (D) in a T cell-free stimulation condition. Naive B cells were cultured in 12-well (1×10^6 /well) plates in the presence of anti-CD40 and cytokines (IL-2, IL-4, and IL-10) for 5 days. CD4⁺CD25⁺CD69⁻ Tregs (1×10^6 /well) were added to the cultures of B cells. RT-PCR was performed to detect the expression of AID, and Ig productive and circle transcripts. DC-PCR was performed to detect Ig switch circles (by-products of CSR). Freshly isolated CD38⁺ GC-B cells (GC) were examined for a positive control. Naive B cells (NV) were stimulated in the T cell-free condition to induce CSR and Ig production. Representative data from three independent experiments are shown.

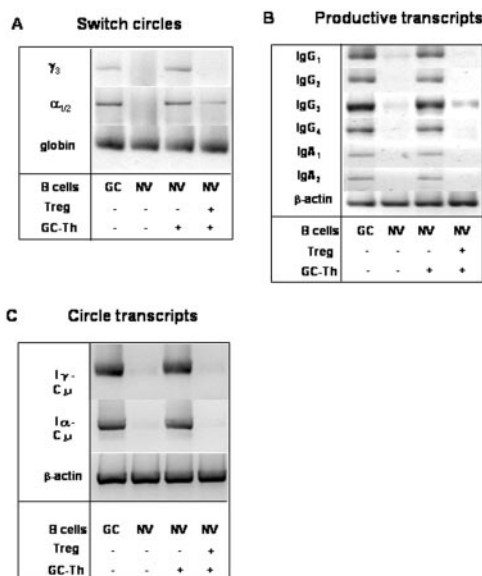


FIGURE 4. Tregs suppress the B cell CSR induced by Th cells. Tregs were examined for their effects on generation of switch circles (A), expression of productive transcripts (B), and expression of circle transcripts (C) in B cells stimulated by GC-Th cells. Naive B cells (NV) were cultured in 12-well (1×10^6 /well) plates with or without CD57⁺ GC-Th cells (1×10^6 /well) for 5 days. CD4⁺CD25⁺CD69⁻ Tregs (1×10^6 /well) were added to the cultures of B cells and CD57⁺ GC-Th cells. SEB (a superantigen) was added to culture for T cell activation. CD38⁺ GC-B cells (GC) were examined for a positive control. Representative data from three independent experiments are shown.

CXCR5 but down-regulate T cell area chemokine receptor CCR7 (14). Second, it has been unknown whether Foxp3⁺ Tregs can directly suppress B cells or whether they have to suppress T cells to indirectly inhibit B cell response. We showed evidence that Tregs can directly suppress B cells without needing to suppress Th cells. Third, the direct suppression of B cells by Tregs was accompanied by reduced Ig CSR. Our results suggest that these follicular Tregs can directly suppress B cells independently of Th cells for effective suppression of B cell response. They can also directly suppress Th cells. This versatile suppressive capacity of Tregs is thought to be important to efficiently limit B cell responses and to maintain immune tolerance in Ig response. Our findings are consistent with the fact that Treg number or activity is significantly decreased in a number of autoimmune or infectious diseases with aberrant Ig responses (7–9).

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Disclosures

The authors have no financial conflict of interest.

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