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Dendritic Cells and B Cells Cooperate in the Generation of $CD4^+CD25^+FOXP3^+$ Allogeneic T Cells

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ABSTRACT

Background. $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) play an essential role in immune tolerance, suppressing responses against self-antigens. Additionally, Treg play an important role in maintaining immunosuppression to alloantigens as well as to other antigens. It is well known that in the gut, a subset of dendritic cells produces retinoic acid (RA), which together with transforming growth factor (TGF-β) is able to differentiate naïve T cells into Treg. The aim of this study was to establish the role of antigen-presenting cells (APC) in the differentiation of allogeneic Tregs under the effect of RA and TGF-β.

Methods. Splenic $CD4^+CD25^-$ naïve T cells from C57BL/6 mice were co-cultured with splenic CD11c-enriched APC from Balb/c mice in the presence of TGF-β, RA, and interleukin (IL-2). After 6 days of culture, cells were analyzed for the expression of Foxp3 by flow cytometry. Additionally, we investigated the role of B cells and dendritic cells (DCs) and their stimulatory capacity in the generation of Tregs.

Results. Our results showed that co-culture of naive T cells with the appropriate level of stimulation by APC in the presence of TGF-β, RA, and IL-2 provided a new powerful approach to generate allogeneic Treg cells. We demonstrated that although B cells and DCs can generate Tregs by themselves, a mixure of both APC improved their capacity to efficiently generate Tregs. Also, we observed that although the addition of IL-2 to the cultures was not crucial to generate Tregs, it was required to optimize their expansion and cell survival.

IMMUNOREGULATION or suppression has long been proposed to play a role in transplantation. Since the early 1970s, various types of regulatory T cells (Treg) have been documented in transplant models.[1] In recent years, progress in characterizing many of these cell types has been remarkable, most notably by the discovery of the transcription factor Foxp3 and its role in Treg function. Naturally occurring $CD4^+CD25^+Foxp3^+$ Tregs (nTregs) represent 5%–10% of peripheral CD4 T cells. Recent studies in the mouse have shown that these cells are critical regulators of immune tolerance.[2–4]

Furthermore, it has been established that under specific conditions naïve $CD4^+CD25^-$ T cells can be converted into $Foxp3^+$ Treg (inducible Tregs); several conversion/expansion protocols for these cells have been described.[5,6] Such protocols usually involve T-cell stimulation under specific cytokine conditions that include exogenous or endogenous transforming growth factor (TGF-β).[7–10] The Treg population generated is usually analyzed for Foxp3 expression and the ability to inhibit proliferation of naïve T cells in vitro. More important, however, several studies have shown that ex vivo generated $Foxp3^+$ cells can regulate T-cell responses in vivo after adoptive transfer into immunodeficient recipients.[8,11–15] Although ex vivo generated/expanded Treg may have significant potential in transplantation, where they could be used as a donor-reactive cellular therapy, the success of this approach depends on several

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Supported by FONDECYT grants 1060834, 1060253, 1080416 Universidad Andres Bello grant DI-08-08/I and PFB-16 fron CONICYT, Chile.

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0041-1345/10/$–see front matter
doi:10.1016/j.transproceed.2009.12.044

factors such as generating Treg in sufficient numbers, the preservation of Foxp3 expression, their in vivo survival after infusion, their in vivo migration to sites of alloreactivity, and, most important, their ability to suppress rejection in an alloantigen-specific manner.[16]

Although the de novo generation of Foxp3$^+$ Treg requires TGF-β, the presence of IL-6 and IL-21 have been shown to act as switch factors that drive the development of the pro-inflammatory IL-17–secreting T helper 17 (Th17) cells at the expense of Treg cell generation. In addition, vitamin A metabolites, including retinoic acid (RA), by binding to their RA-related nuclear receptors play important roles in several biological processes. In this regard, it has recently been described that the in vitro addition of RA enhances TGF-β signaling by increasing the expression and phosphorylation of Smad3, resulting in increased Foxp3 expression even in the presence of IL-6 or IL-21, thus suppressing the differentiation pathway to Th17 cells.[17,18]

New evidence has demonstrated that B cells preferentially induce the expansion of alloreactive mouse Treg cells.[19] We have demonstrated herein that B cells are an important factor in the generation of inducible Tregs, acting in synergy with DC in the presence of IL-2, TGF-β, and RA to generate de novo allogeneic CD4$^+$CD25$^+$Foxp3$^+$ T cells.

MATERIALS AND METHODS

Generation of CD4$^+$CD25$^+$Foxp3$^+$ Cells

CD4$^+$CD25$^-$ naïve T cells were obtained from spleens of 6- to 8-week-old, pathogen-free, female C56BL/6 mice. First, naïve T cells were obtained by negative selection using a MACS CD4$^+$ T-cell isolation kit (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. Then these cells were stained with an anti-CD25 antibody conjugated to R-phycoerythrin (PE; eBioscience, San Diego, Calif) followed by MACS beads anti-PE to separate CD25$^+$ cells. The resulting CD4$^+$CD25$^-$ cells were ≥97% pure as shown by flow cytometry analysis.

Partially purified APCs were obtained from spleens of 6- to 8-week-old Balb/c mice using anti-CD11c MACS beads; this fraction contained ≥60% DC as determined by flow cytometry. To obtain purified CD11c$^+$ DC, the enriched CD11c$^+$ APCs were subjected to a second round of CD11c$^+$ microbead selection, obtaining an >90% pure DC fraction. B cells were obtained from the same partially purified fraction with an anti-B220 antibody plus MACS beads anti-rat immunoglobulin (Ig)G to obtaining a ≥95% purity.

The purified T cells and APCs were cultured at a 7:1 ratio in U-bottom, 96-well plates (Corning Coster, Corning, NY) with or without exogenous TGF-β (2 ng/mL; eBioscience) retinoic acid (10 nmol/L; Sigma Aldrich, St. Louis, Mo) and IL-2 (10 μg/mL; eBioscience) for 6 days. Live cell numbers per culture were counted by trypan blue exclusion.

Flow Cytometry

To analyze the number of Tregs generated after culture, the cells were first stained with anti-CD4 and anti-CD25, and after fixation and permeabilization, they were stained with anti-Foxp3 (eBioscience) or the corresponding isotype control reagent. Cells were analyzed using a FACSCanto II (Becton Dickinson).

RESULTS AND DISCUSSION

Naïve CD4$^+$CD25$^-$ T cells from C57BL/6 mice were cultured with allogeneic spleen CD11c-enriched APC with or without exogenous TGF-β, RA, and IL-2. We evaluated at day 6 (Fig 1), the percentage of Foxp3$^+$ T cells by flow cytometry, determining that spleen-enriched APC efficiently differentiated naïve T cells into Foxp3$^+$ cells, when they were in the presence of TGF-β and RA (81%). In the absence of RA, TGF-β induced a lower percentage of Foxp3$^+$ cells (14%), and in the absence of exogenous cytokines fewer than 3% of the cultured cells expressed Foxp3$^+$.

Although IL-2 was not necessary for the induction of Foxp3$^+$ cells, the inclusion of this cytokine in the cultures allowed a 10-fold expansion of Tregs compared with the mixture of TGF-β and RA alone, even though the percentage of Foxp3$^+$ cells was slightly lower (63%), possibly owing to the expansion of the remaining population of effector T cells in the cultures (Fig 1). It is well known that IL-2 is essential for T-cell proliferation, so its addition to the cultures was necessary to maintain Foxp3 expression and cell survival in an allogeneic model, in contrast with a transgenic model where endogenous IL-2 has been shown to be sufficient for Treg survival.[6]

Seeking to determine the optimal condition for the generation of Treg cells, we co-cultured naïve T cells with CD11c-enriched APCs at different ratios in the presence of TGF-β, RA, and IL-2, and determined the level of Foxp3 expression in the resulting cells. When cells were cultured at a 1:5 ratio (APCs to T cells), the percentage of Foxp3$^+$ cells was slightly higher compared with a 1:10 ratio (76.6% vs 68%, respectively; Fig 2). However, the absolute number of cells recovered was 4 times higher in the 1:10 ratio, which represented an advantage for future strategies to generate allospecific Tregs for organ transplantation. These results agree with previous published data where a low level of antigen stimulation was necessary for optimal Foxp3 induction.[6,20]

Recent evidence has shown that B cells are capable of expanding Tregs in the presence of co-stimulatory molecules plus IL-2.[19,21] When we analyzed the APC populations that was used for the Treg generation, we observed that 60% of the cells were CD11c$^+$ with an immature phenotype, and a low expression of CD80 and CD86 molecules (data not shown), but an important proportion of the cells were CD11c$^-$. These elements were identified as mainly being B cells by their expression of CD19 and B220 (20%).

To determine the contribution of these B cells to the induction of Foxp3$^+$ T cells, we co-cultured an isolated B-cell population with naïve T cells in the presence of TGF-β, RA, and IL-2 at a 1:7 ratio (B to T cells). Figure 3 shows that B cells alone were not as efficient for the generation of Tregs (33%) as partially purified CD11c-enriched APCs (70%). Similarly, purified (>90%) CD11c$^+$ DCs induced a percentage of Foxp3$^+$ T cells comparable

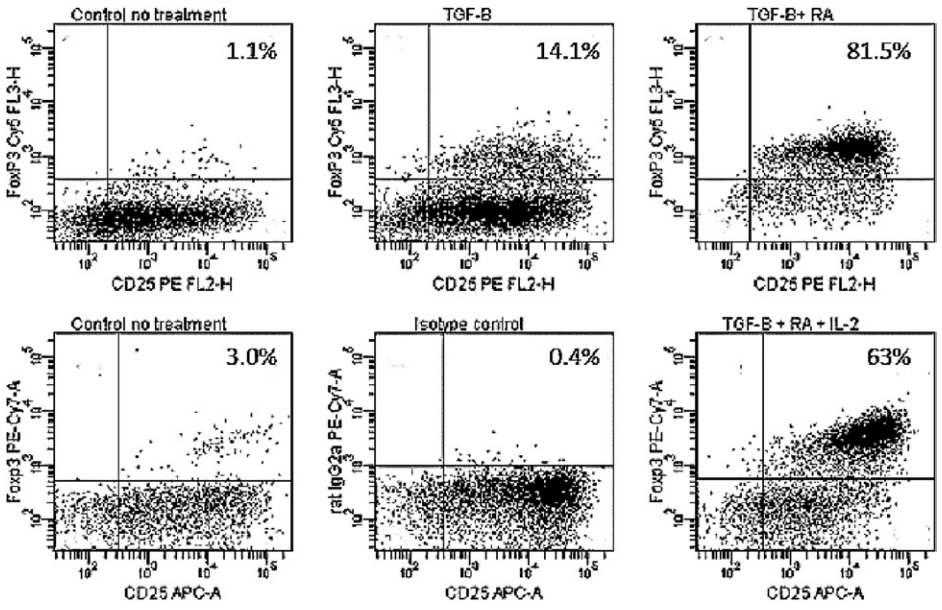

Fig 1. Retinoic acid synergizes with TGF-β to induce Foxp3 expression in naïve T cells cultured with allogeneic splenic APC. Naïve T cells cultured with allogeneic APCs were incubated in the presence or absence of TGF-β and RA. At day 6, the cultures were analyzed for the expression of Foxp3 in a gate of CD4$^+$ T cells. The results are representative of 3 independent experiments.

with B cells alone (data not shown). Interestingly, isolated B cells induced a higher proportion of Tregs in the absence of cytokines than the partially purified APC fraction. Taken together, these results demonstrate that optimal Treg induction occurs when both populations of APCs are present. Additionally, we observed that stimulation of naïve T cells with B cells alone produced fewer effector CD4$^+$CD25$^+$ T cells compared with stimulation with partially purified APCs, which agrees with the fact that B cells display antigens on their surface but do not activate T cells owing to the absence of co-stimulatory molecules. Our own preliminary data showed that Foxp3$^+$ cells generated with allogeneic, partially purified APCs showed regulatory properties, because they suppressed the proliferation of effector T cells in vitro. This strategy to produce allogeneic Tregs is being further studied using in vivo experiments to determine their capacity to migrate to the inflamed tissue and control immune rejection of the allograft.

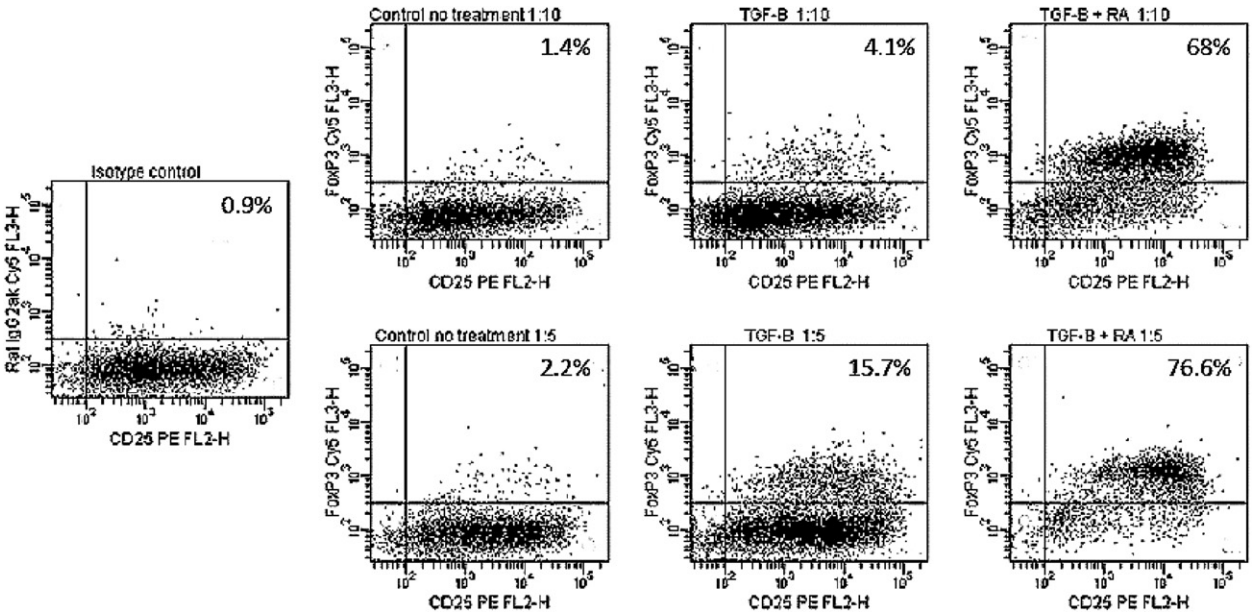

Fig 2. Different ratios of APC generate different levels of CD4$^+$CD25$^+$Foxp3$^+$ cells. Naïve T cells were cultured with allogeneic APC at different ratios in the presence or absence of TGF-β and RA. At day 6, the cultures were analyzed for the expression of Foxp3 in a gate of CD4$^+$ T cells. The result is representative of 3 independent experiments.

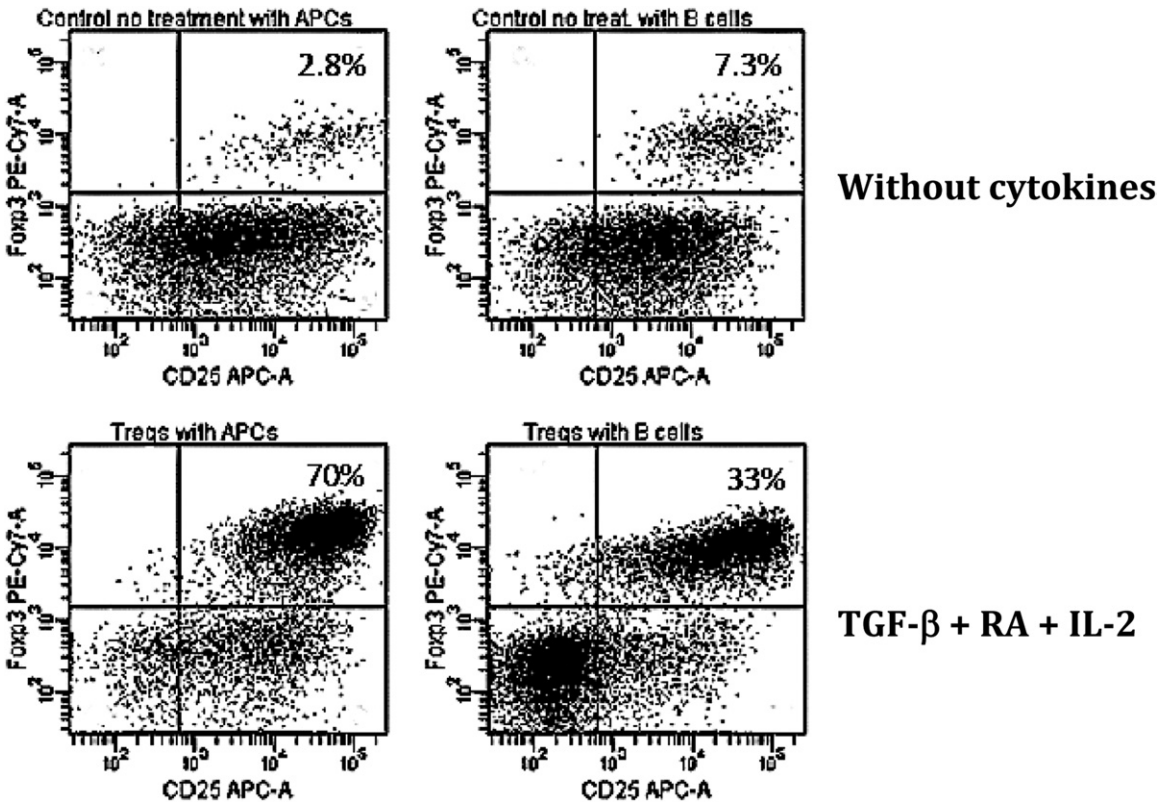

Fig 3. APCs are better inducers of $CD4^+CD25^+Foxp3^+$ cells than B cells. Naïve T cells were cultured with allogeneic APCs or B cells in the presence or absence of TGF-β and RA. At day 6, the cultures were analyzed for the expression of Foxp3 in a gate of $CD4^+$ T cells. The result is representative of 3 independent experiments.

Our data highlighted the cooperative roles of DC and B cells concurrent with low levels of antigen stimulation plus RA, TGF-β and IL-2 to optimally induce $Foxp3^+$ T cells. This strategy may provide a new approach in our objective to generate allospecific Treg for organ transplantation.

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