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Transforming Growth Factor-β and All-Trans Retinoic Acid Generate Ex Vivo Transgenic Regulatory T Cells With Intestinal Homing Receptors

C. Moore, D. Sauma, J. Morales, M.R. Bono, M. Rosemblatt, and J.A. Fierro

ABSTRACT

$CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) mediate immunologic self-tolerance and suppress immune responses. In the gut, a subset of dendritic cells is specialized to induce Treg in a transforming growth factor-β (TGF-β)- and retinoic acid (RA)-dependent manner. The aim of this study was to establish if RA synergizing with TGF-β induced antigen specific $CD4^+$ $CD25^{high}$ $Foxp3^+$ Treg portraying gut homing receptors. Splenic $CD4^+CD25^-$ $Foxp3^-$ naïve T cells from DO11.10 mice were cocultured with splenic $CD11c^+$ dendritic cells from Balb/c mice in the presence of TGF-β, RA, and low levels of an antigenic peptide. After 5 days of culture, cells were analyzed for the expression of Foxp3 and the gut homing receptors CCR9 and $\alpha4\beta7$.

The number of $Foxp3^+$ T cells generated with TGF-β and RA was at least 3 times higher than in the cultures with TGF-β alone and 15 times higher than in controls without exogenous cytokines. Also, supplementation of the cultures with RA induced the expression of the intestinal homing receptors CCR9 and $\alpha4\beta7$. Our results showed that coculture of naïve T cells with antigen-presenting cells in the presence of TGF-β and RA represents a powerful approach to generate Treg with specific homing receptors.

REGULATORY T CELLS (Treg) play a critical role to inhibit deleterious responses against self-antigens. Harnessing Treg faculties for potential adoptive cell therapy is a promising approach to promote donor-specific transplant acceptance. Therefore, the generation of a significant number of Treg would be highly desirable to develop therapies that achieve long-term transplantation survival. $Foxp3^+$ $CD25^+CD4^+$ Treg produced in the thymus (natural Treg) can also differentiate from peripheral $Foxp3^-CD4^+$ T cells.[1] Splenic dendritic cells (DC) efficiently differentiate $Foxp3^+$ Treg from naïve T cells in the presence of low doses of antigenic peptide. The addition of transforming growth factor-β (TGF-β) is necessary to differentiate Treg ex vivo, while exogenous interleukin (IL)-2 is not essential for the generation of Treg, since this cytokine is produced endogenously by the T cells.[2]

Previous work has shown that the vitamin A metabolite, retinoic acid (RA), enhances the expression of $\alpha4\beta7$ and CCR9 on T cells upon activation imprinting with intestinal tropism.[3,4]

The generation of $Foxp3^+CD25^+CD4^+$ using splenic DC in the presence of TGF-β and RA, without exogenous IL-2, was our goal to develop a strategy that would allow high yields of Treg. The aim of this study was to generate TCR transgenic regulatory T cells with gut homing potential under conditions of low-peptide stimulation requiring only the addition of exogenous TGF-β and RA.

MATERIALS AND METHODS

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

$CD4^+CD25^-$ T cells were obtained from 6- to 8-week-old pathogen-free, female DO11.10 mice and $CD11c^+$ cells were isolated from 6- to 8-week-old Balb/c mice.

From the Immunology Laboratory (C.M., D.S., M.R.B., M.R.), Faculty of Science, Universidad de Chile, Chile; Faculty of Biological Sciences (C.M., M.R.), Universidad Andrés Bello, Santiago, Chile; Transplantation Unit (J.M., J.A.F.), Clínica Las Condes, Santiago, Chile; and Fundación Ciencia para la Vida (M.R.), Santiago, Chile.

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Address reprint request to Dr Juan Alberto Fierro, Clínica Las Condes, Lo Fontecilla 441, Santiago, Chile. E-mail: afierro@clc.cl

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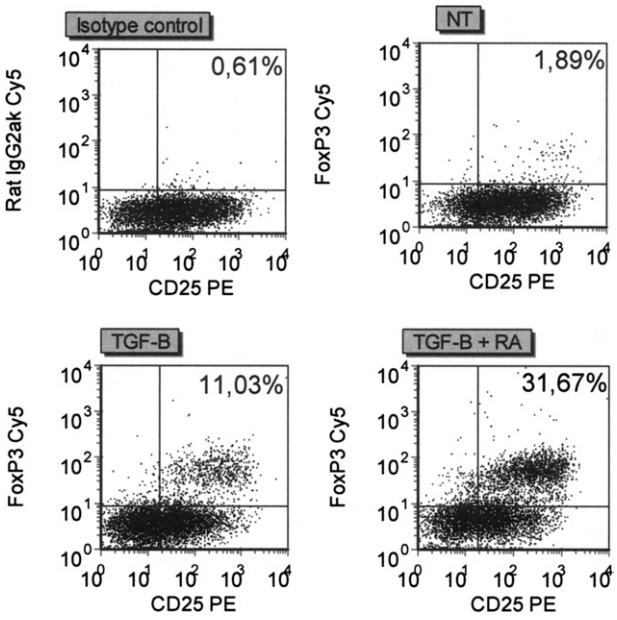

Fig 1. Retinoic acid (RA) synergizes with transforming growth factor-β (TGF-β) to induce Foxp3 expression in naive T cells cultured with splenic dendritic cells (DC). Naïve T cells cultured with splenic DC were incubated in the presence or absence (NT) of TGF-β and RA. At day 5, the cultures were analyzed for the expression of Foxp3 in $CD4^+$ T cells. The result is representative of three independent experiments.

$CD4^+$ $CD25^-$ T cells (>90%) were negatively selected by MACS® beads from spleen cell suspensions (Miltenyi Biotech, Gladbach, Germany). Spleen $CD11c^+$ DC (>90%) were selected with anti-CD11c MACS beads. The purified T cells and DC were cultured at a 1:1 ratio in 96-well plates (Corning Coster, Corning, NY, USA) with or without exogenous TGF-β (2 ng/mL; eBioscience, San Diego, Calif, USA) and RA (10 nmol/L; Sigma Aldrich, St Louis, Mo, USA), in the presence of ovalbumin $peptide_{323–336}$ (0.03 μg/mL). Live cell numbers per culture were counted by trypan blue exclusion.

Flow Cytometry

To analyze the number of Treg generated after culture with TGF-β and RA, the cells were first stained with anti-CD4, then anti-CD25, and following fixation and permeabilization, with anti-Foxp3 (eBioscience) or its isotype control. In parallel, cultured cells were analyzed for the expression of gut homing receptors using anti-CCR9 and anti-$\alpha4\beta7$ antibodies (Becton Dickinson, Bioscience, San Diego, Calif, USA). Cells were analyzed using a FACSCan (Becton Dickinson).

RESULTS

$Foxp3^-CD25^-CD4^+$ T cells from DO11.10 mice were cultured with spleen $CD11c^+$-enriched DC, with or without exogenous TGF-β, RA, and OVA peptide. At day 5, we assessed the percentage of $Foxp3^+$ T cells in the cultures, determining that spleen DC had efficiently differentiated naive T cells into $Foxp3^+$ cells in the presence of TGF-β and low doses of OVA peptide, even in the absence of IL-2 (Fig 1). In the presence of only TGF-β, we obtained 10% to 12% $Foxp3^+$ T cells. We also observed an increase in the number of $Foxp3^+$ regulatory T cells using the combination of TGF-β and RA, where the yield of $Foxp3^+$ cells was always 30% to 35% of $CD4^+$ T cells. In contrast, $Foxp3^-CD25^-CD4^+$ T cells cultured in complete absence of exogenous cytokines only expressed 1% to 2% $Foxp3^+$ cells.

These Treg were further analyzed for their expression of gut homing receptors CCR9 and $\alpha4\beta7$. As shown in Fig 2, CCR9 and $\alpha4\beta7$ were not expressed on T cells cultured in the absence of exogenous cytokines or in the presence of only TGF-β. The inclusion of RA to the cultures enhanced CCR9 and $\alpha4\beta7$ expression on T cells. These data agree

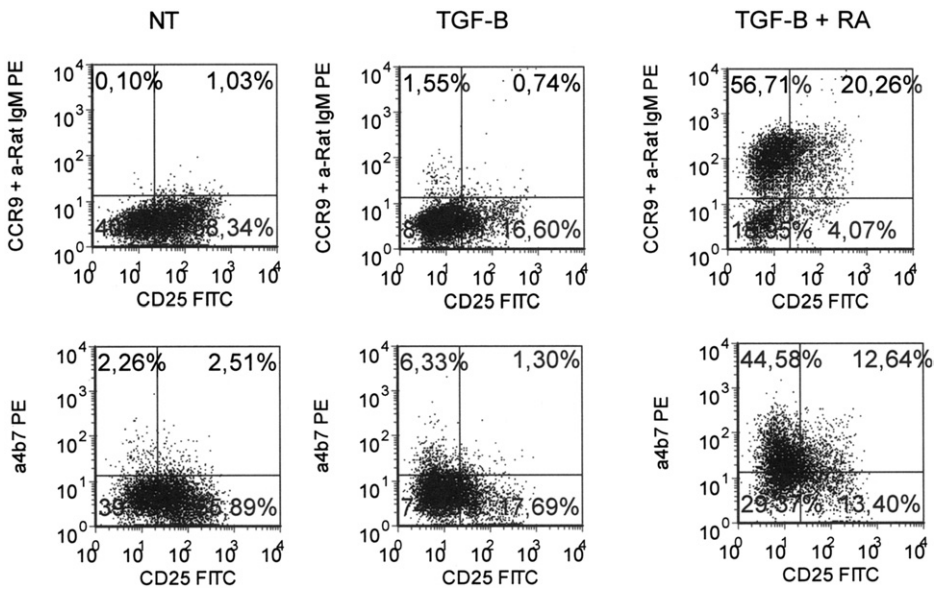

Fig 2. Retinoic acid (RA) induces the expression of gut-homing receptors on regulatory T cells. Naïve T cells cultured with splenic dendritic cells were incubated in the presence or absence (NT) of transforming growth factor-β (TGF-β) and RA. At day 5, the cultures were analyzed for the expression of CCR9 and $\alpha4\beta7$ by flow cytometry. The result is representative of three independent experiments.

with previous work showing that CCR9 and $\alpha 4\beta 7$ expressions are dependent on the presence of retinoic acid.

DISCUSSION

We used spleen DC, which show an immature phenotype at steady state and contribute to deletional tolerance in the periphery. Under low levels of peptide stimulation and in the presence of TGF-β, splenic DC induce Foxp3 in naïve T cells, but when RA was added to the cultures, the number of cells that expressed Foxp3 increased substantially. The addition of exogenous IL-2 was not necessary to generate $Foxp3^+$ $CD25^+CD4^+$ T cells.

Taken together, our data suggested that low levels of stimulation, RA, and TGF-β are necessary to induce $Foxp3^+$ T cells that have potential intestinal tropism. This strategy may provide a new approach to generate allospecific Treg for organ transplantation.

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