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TITLE:

In Vivo Augmentation of Gut-Homing Regulatory T cell Induction

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gut-homing regulatory T cells, 25-hydroxyvitamin D 1α-hydroxylase, cytochrome P450 family 27 subfamily B member 1, 1,25-dihydroxyvitamin D, foxp3, dendritic cells, retinaldehyde dehydrogenase 2, c-c chemokine receptor type 9

SUMMARY:

Here we present a protocol for in vivo augmentation of gut-homing regulatory T cell induction. In this protocol, dendritic cells are engineered to locally produce high concentrations of the active vitamin D (1,25-dihydroxyvitamin D or 1,25$[OH]_2$D) and the active vitamin A (retinoic acid or RA) de novo.

ABSTRACT:

Inflammatory bowel disease (IBD) is an inflammatory chronic disease in the gastrointestinal tract (GUT). In the United States, there are approximately 1.4 million IBD patients. It is generally accepted that a dysregulated immune response to gut bacteria initiates the disease and disrupts the mucosal epithelial barrier. We recently show that gut-homing regulatory T (Treg) cells are a promising therapy for IBD. Accordingly, this article presents a protocol for in vivo augmentation of gut-homing Treg cell induction. In this protocol, dendritic cells are engineered to produce locally high concentrations of two molecules de novo, active vitamin D (1,25-dihydroxyvitamin D or 1,25$[OH]_2$D) and active vitamin A (retinoic acid or RA). We chose 1,25$(OH)_2$D and RA based on previous findings showing that 1,25$(OH)_2$D can induce the expression of regulatory molecules (e.g., forkhead box P3 and interleukin-10) and that RA can stimulate the expression of gut-homing

receptors in T cells. To generate such engineered dendritic cells, we use a lentiviral vector to transduce dendritic cells to overexpress two genes. One gene is the cytochrome P450 family 27 subfamily B member 1 that encodes 25-hydroxyvitamin D 1α-hydroxylase, which physiologically catalyzes the synthesis of $1,25(OH)_2D$. The other gene is the aldehyde dehydrogenase 1 family member A2 that encodes retinaldehyde dehydrogenase 2, which physiologically catalyzes the synthesis of RA. This protocol can be used for future investigation of gut-homing Treg cells in vivo.

INTRODUCTION:

Inflammatory bowel disease (IBD) is an inflammatory chronic disease in the gastrointestinal tract (GUT). In the United States, there are approximately 1.4 million IBD patients. It is generally accepted that a dysregulated immune response to gut bacteria initiates the disease and disrupts the mucosal epithelial barrier[1,2]. For this reason, currently available U.S. Food and Drug Administration (FDA)-approved drugs inhibit the functions of inflammatory mediators or block the homing of immune cells into the gut. However, the inflammatory mediators and immune cells that are targeted are also necessary for immune defenses. As a result, the inflammatory mediator inhibitors compromise systemic immune defense and the immune cell homing blockers weaken gut immune defense, both of which can lead to severe consequences[3,4]. In addition, the immune cell homing blockers can also block the homing of regulatory T (Treg) cells into the gut and hence can worsen the already compromised gut immune tolerance in IBD patients. Furthermore, blocking of Treg cell homing into the gut may also lead to systemic immune suppression due to the accumulation of Treg cells in the blood[5]. Finally, inhibitors and blockers function transiently and, thereby, require frequent administrations. Frequent administration of these inhibitors and blockers may further exacerbate the untoward side effects.

Recently, we proposed a novel strategy that can potentially mitigate or even eliminate the side effects associated with current drugs for IBD treatment[6]. This strategy augments the induction of gut-homing Treg cells in peripheral lymphoid tissues[6]. The rationale of this strategy is that gut-homing Treg cells specifically home to the gut and hence will not compromise systemic immune defenses. In addition, since Treg cells can potentially form memory[7,8], gut-homing Treg cells can potentially provide a stable control of the chronic gut inflammation in IBD patients and, thereby, treatment should not need to be administered as frequently. Furthermore, since this strategy augments the induction of gut-homing Treg cells in vivo, it does not have the concern of in vivo instability in a highly proinflammatory environment that is associated with adoptive transfer of in vitro generated Treg cells[9,10]. In this regard, in vitro generated Treg cells are one of the proposed strategies for the treatment of autoimmune diseases[11–13] and transplant rejection[14,15]. Finally, in this strategy, dendritic cells (DCs) are engineered to produce locally high concentrations of two molecules de novo: active vitamin D (1,25-dihydroxyvitamin D or $1,25[OH]_2D$) and active vitamin A (retinoic acid or RA). We chose $1,25(OH)_2D$ and RA because $1,25(OH)_2D$ can induce the expression of regulatory molecules (e.g., forkhead box P3 [foxp3] and interleukin-10 [IL-10])[16,17] and that RA can stimulate the expression of gut-homing receptors in T cells[18]. Because both $1,25(OH)_2D$ and RA can also tolerize DCs[28,29], we reason that the engineered DCs will be stably maintained in a tolerogenic status in vivo and hence circumvent the in vivo instability concerns that are associated with in vitro generated tolerogenic DCs (TolDCs)[19,20,21]. In this respect, TolDCs

are also one of the proposed strategies for in vivo augmentation of Treg cell functions[19,20,21]. To support our reasoning, we have shown that the engineered DCs, upon in vivo delivery, can augment the induction of gut-homing Treg cells in peripheral lymphoid tissues[6].

An additional advantage of our proposed strategy is that $1,25(OH)_2D$ also has other functions that can potentially benefit IBD patients. These other functions include the ability of $1,25(OH)_2D$ to stimulate the secretion of antimicrobials[22] and to suppresses carcinogenesis[23].Infections and cancers are frequently associated with IBD[24,25].

To generate the DCs that can produce locally high concentrations of both $1,25(OH)_2D$ and RA de novo, we use a lentiviral vector to engineer DCs to overexpress two genes. One gene is the cytochrome P450 family 27 subfamily B member 1 (CYP27B1) that encodes 25-hydroxyvitamin D 1α-hydroxylase (1α-hydroxylase), which physiologically catalyzes the synthesis of $1,25(OH)_2D$. The other gene is the aldehyde dehydrogenase 1 family member A2 (ALDH1a2) that encodes retinaldehyde dehydrogenase 2 (RALDH2), which physiologically catalyzes the synthesis of RA[6].

Because in vivo augmentation of gut-homing Treg cell induction is potentially important in the treatment of IBD, in the following protocol we will detail the procedures for the generation of the 1α-hydroxylase-RALDH2-overexpressing DCs (DC-CYP-ALDH cells) that can be used for the future investigation of gut-homing Treg cells in vivo.

PROTOCOL:

All in vivo animal study protocols were reviewed and approved by the Loma Linda University Institutional Animal Care and Use Committee (IACUC) as well as the Animal Care and Use Review Office (ACURO) of the US Army Medical Research and Materiel Command (USAMRMC) of the Department of Defense.

1. Preparation of the lentivirus that expresses both 1α-hydroxylase and RALDH2 (lenti-CYP-ALDH virus)

1.1. Day 0: In the early morning, prepare 5 x 10^5 cells/mL of 293T cells in the CM-10-D cell culture medium.

1.2. Seed 20 mL/plate in 150 mm x 25 mm culture dishes. Culture the cells at 37 ˚C and 5% CO_2 for 24 h. Confluency will reach ~80–90% after 24 h.

1.3. Day 1: Make 2x HBS (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 7.1). Aliquot and store at -20 ˚C.

1.4. Make 2 M $CaCl_2$ and store at room temperature.

1.5. For each 150 mm x 25 mm culture dish, prepare a DNA precipitation mixture in a sterile 50 mL culture tube. First, add 1,620 µL of 2x HBS, 9.5 µg of PMD2G (VSVG), 17.5 µg of pCMVR8.74 (Capsid), 27 µg of Lenti-CYP-ALDH plasmid (a lentiviral vector that carries both CYP27B1 and

ALDH1a2). Second, add H_2O to a final volume of 3,037.5 µL.

1.6. In the last, add 202.5 µL of 2 M $CaCl_2$ dropwise while mixing the solution to final concentrations of 1x HBS and 125 mM $CaCl_2$. $CaCl_2$ must be the last to be added. Leave the transfection mixtures at room temperature for 20 min with occasional mixing. For multiple 150 mm x 25 mm culture dishes, scale up accordingly.

1.7. Add the 3,240 µL of DNA precipitation mixture dropwise to one 150 mm x 25 mm culture dish containing the 293T cells. Gently swirl the culture dish side to side while adding the DNA precipitation mixture. Incubate the dish at 37 ˚C and 5% CO_2 for 24 h.

1.8. Day 2: Remove the calcium phosphate transfection solution, wash gently with 1x PBS, and refeed the cell culture with CM-4-D cell culture medium. Incubate the cells at 37 ˚C and 5% CO_2 for 24 h.

1.9. Day 3: Harvest the supernatants in sterile storage bottles and store at 4–8 ˚C. Replenish the cell culture with fresh CM-4-D cell culture medium. Culture the cells at 37 ˚C and 5% CO_2 for another 24 h.

1.10. Day 4: Harvest the supernatants into sterile storage bottles. Filter the supernatants from Day 3 and Day 4 through a 0.45 µm filter. To concentrate the VSVG pseudotype virus, transfer the supernatants into centrifuge tubes and spin at 4,780 x *g* for 24 h at 4 ˚C.

1.11. Day 5: Pour the supernatants off the pellets (the pellet is often visible) and allow the tubes to drain on a paper towel in a sterile biosafety cabinet for several minutes.

1.12. Resuspend the pellets by pipetting with sterile PBS containing 5% glycerol. The volume for resuspension will be 33.3 µL per 150 mm x 25 mm culture dish.

1.13. Aliquot 200 µL/tube and store at -80 °C. Make a separate aliquot of 50 µL/vial for titration purposes. Titers should be within the range of 10^8–10^9 transducing units (TUs)/mL.

1.14. Add bleach into the culture dishes and discard them.

NOTE: These transfected cells are the biggest safety concern in the protocol since all lentiviral elements are expressed in the cells.

2. Generation of bone marrow derived DCs (BMDCs)

2.1. Harvest tibias and femurs from 4–5 Balb/c mice into a 50 mL sterile polypropylene tube containing the culture medium[6].

2.2. Transfer the tibias and femurs into a 100 mm x 20 mm culture dish containing the culture medium. Remove tissues such as muscles from the tibias and femurs using dissecting scissors and

forceps.

2.3. Cut both ends of the tibias and femurs to expose the bone marrow cavity.

2.4. Draw 10 mL culture medium in a 10 mL syringe attached to a 30 G needle.

2.5. Carefully insert the needle into the bone marrow cavity and flush the bone marrow into a clean 50 mL sterile polypropylene tube. Repeat this procedure if necessary, to ensure that the bone marrow has been completely flushed out.

2.6. Pellet the bone marrow cells by centrifugation (400 x *g*) at 2–8 ˚C for 5 min. Aspirate the supernatant.

2.7. Resuspend the pellet with 5 mL of 1x red blood cell lysis buffer.

2.8. Incubate the cells at room temperature for 4–5 min with occasional shaking.

2.9. Stop the reaction by adding 30 mL of culture medium.

2.10. Pellet the cells by centrifugation (400 x *g*) at 2–8 ˚C for 5 min. Resuspend the cells in 10 mL of CM-10-R cell culture medium. If needed, the cells can be passed through a 40 µm cell strainer to remove debris.

2.11. Perform a cell count and adjust the cells to 1 x 10^6 cells/mL using CM-10-R cell culture medium.

2.12. Add recombinant murine granulocyte/macrophage colony-stimulating factor (GM-CSF, final concentration = 100 U/mL) and murine interleukin-4 (IL-4, final concentration = 10 U/mL).

NOTE: The stock solutions of GM-CSF and IL-4 are stored at -80 ˚C at 100,000 U/mL (1,000 x) and 10,000 U/mL (1,000 x), respectively.

2.13. Distribute the cells into 6 well culture plates at 4 mL/well and culture the cells at 37 ˚C and 5% CO_2 for 48 h.

2.14. Remove nonadherent cells with gentle pipetting.

2.15. Add fresh CM-10-R cell culture medium containing the GM-CSF (100 U/mL) and IL-4 (10 U/mL). Culture the cells for another 48 h.

2.16. Harvest nonadherent cells (BMDCs) for transduction by the lenti-CYP-ALDH virus.

3. Transduction of DCs with lenti-CYP-ALDH virus to generate DC-CYP-ALDH cells

3.1. Prepare 1 x 10^6 DCs/well in a total volume of 0.5 mL of CM-10-R cell culture medium containing 50 µL of virus and 8 µg/mL protamine in a 6 well culture plate.

3.2. Culture the cells at 37 ˚C and 5% CO_2 for 24 h.

3.3. Replace the medium with fresh CM-10-R cell culture medium and culture the cells at 37 °C and 5% CO_2 for another 24 h. At this time point, the enzymatic activities of 1α-hydroxylase and RALDH2 can be evaluated (see step 4). If necessary, repeat steps 3.1–3.3 for a second transduction.

3.4. Add lipopolysaccharide (100 ng/mL) and culture the cells for 24 h.

3.5. Harvest the cells (DC-CYP-ALDH cells) for experiments.

4. Evaluation of the overexpressed 1α-hydroxylase and RALDH2 in DC-CYP-ALDH cells

4.1. Evaluation of the overexpressed 1α-hydroxylase in DC-CYP-ALDH cells

4.1.1. Seed 1.0 x 10^6 DC-CYP-ALDH cells/well in 2 mL of CM-10-R cell culture medium into 12 well culture plates.

4.1.2. Add 25(OH)D to the final concentration of 2.5 µM. Incubate the DC-CYP-ALDH cells for 24 h.

4.1.3. Harvest the supernatants and store at -20 ˚C.

4.1.4. Assay for 1,25$(OH)_2$D concentrations in the supernatants using a commercially available radioimmunoassay (RIA) (see **Table of Materials**).

4.1.5. Determine the expression of 1α-hydroxylase in DC-CYP-ALDH cells by fluorescence activated cell sorting (FACS) using an anti-CYP27B1 antibody.

4.2. Evaluation of the overexpressed RALDH2 in DC-CYP-ALDH cells

NOTE: The expression and activity of the overexpressed RALDH2 are determined using a commercial aldehyde dehydrogenase (ALDH) detection kit (see **Table of Materials**).

4.2.1. Seed 1 mL of DC-CYP-ALDH or control cells (1 x 10^5 cells/mL in assay buffer) in each of two 5 mL tubes (one labeled as “Control” and the other labeled as “Test”).

4.2.2. Add 10 µL of diethylaminobenzaldehyde (DEAB) solution (1.5 mM in 95% ethanol) to each of the control tubes and mix immediately. DEAB is a RALDH2 inhibitor.

4.2.3. Add 5 µL of activated RALDH fluorescence substrate (300 µM) to the Control and Test tubes

and mix immediately.

4.2.4. Incubate the tubes for 45 min.

4.2.5. Pellet the cells by centrifugation at 400 x *g* at 2–8 ˚C for 5 min. Aspirate the supernatants.

4.2.6. Reconstitute the cells in 200 µL of assay buffer.

4.2.7. Keep the cells on ice and proceed for analysis by FACS.

REPRESENTATIVE RESULTS:

DC-CYP-ALDH cells expressed significantly increased amount of 1α-hydroxylase. To determine whether DC-CYP-ALDH cells generated from BMDCs expressed a significantly increased amount of 1α-hydroxylase, BMDCs were transduced with the lenti-CYP-ALDH virus to produce bone-marrow-derived DC-CYP-ALDH cells (BMDC-CYP-ALDH cells). Subsequently, the BMDC-CYP-ALDH cells were examined for the expression of 1α-hydroxylase by FACS. Our data showed that the BMDC-CYP-ALDH cells, when compared to the parental BMDCs, displayed enhanced expression of the 1α-hydroxylase (**Figure 1A**). We also determined the enzymatic activity of the 1α-hydroxylase in the BMDC-CYP-ALDH cells. To do so, 1.0 x 10^6 BMDC-CYP-ALDH cells in 2 mL of CM-10-R cell culture medium were added into 12 well culture plates. 25(OH)D was then added to the cell culture at the final concentration of 2.5 µM. The cells were cultured at 37 ˚C and 5% CO_2 for 24 h and the supernatants were harvested for the measurement of 1,25$(OH)_2$D using a radioimmunoassay (RIA). Our data showed that 1,25$(OH)_2$D concentrations in the culture supernatants of the BMDC-CYP cells (BMDCs transduced with lenti-CYP-GFP virus) and the BMDC-CYP-ALDH cells were each approximately 20x higher than those of the parental BMDCs and the BMDC-ALDH cells (BMDCs transduced with lenti-ALDH virus) (**Figure 1B**).

DC-CYP-ALDH cells expressed significantly increased amount of RALDH2. To determine whether BMDC-CYP-ALDH cells expressed a significantly increased amount of RALDH2, a RALDH2 substrate was added into the cell cultures in the presence or absence of the RALDH2 inhibitor diethylaminobenzaldehyde (DEAB) (15 µM). The fluorescent product retained inside the cells was analyzed by FACS. Our data showed that mean fluorescence intensities (MFIs) of the BMDC-CYP-ALDH cells were approximately 6x higher than those of the parental BMDCs, suggesting that the BMDC-CYP-ALDH cells, when compared to the parental BMDCs, expressed significantly enhanced RALDH2 enzymatic activity (**Figure 2A**,**B**).

DC-CYP-ALDH cells augmented the induction of foxp3$^+$CCR9$^+$ gut-homing Treg cells in vitro. To investigate whether DC-CYP-ALDH cells were able to augment the induction of gut-homing Treg cells in vitro, we transduced DC2.4 cells (a bone marrow-derived DC line[26–29]), with lenti-CYP-ALDH virus and generated DC2.4-CYP-ALDH cells. Subsequently, we determined whether the DC2.4-CYP-ALDH cells were able to augment the induction of gut-homing Treg cells in vitro. Accordingly, naive CD4$^+$ T cells were purified from C57BL/6 mice. Purified naive CD4$^+$ T cells at 5 x 10^5 cells/well were then cocultured with either the parental DC2.4 cells (1 x 10^5 cells/well) or the DC2.4-CYP-ALDH cells (1 x 10^5 cells/well) in 24 well culture plates in a serum-free medium in

the presence of an anti-CD3 monoclonal antibody (5 μg/mL) and recombinant human IL-2 (50 U/mL). In addition, 25(OH)D and retinol at various concentrations were also added into the cultures. The cells were incubated at 37 ˚C and 5% CO_2. Five days later, the cells were analyzed by FACS for the expressions of foxp3 and c-c chemokine receptor type 9 (CCR9). Our data showed that in the presence of the substrates, the DC2.4 cells did not significantly change the abundance of foxp3$^+$CCR9$^+$ cells in the CD4$^+$ T cell populations (**Figure 3A**,**B**). In contrast, the DC2.4-CYP-ALDH cells significantly enhanced the abundance of foxp3$^+$CCR9$^+$ cells among CD4$^+$ T cells. In addition, the more 25(OH)D added, the greater the ability of the DC2.4-CYP-ALDH cells to increase the abundance of foxp3$^+$CCR9$^+$ cells among CD4$^+$ T cells. Therefore, our data support that the DC-CYP-ALDH cells can augment the induction of gut-homing Treg cells in vitro.

DC-CYP-ALDH cells augmented the induction of foxp3$^+$CCR9$^+$ gut-homing Treg cells in vivo. To determine whether DC-CYP-ALDH cells could augment the induction of gut-homing Treg cells in vivo, we intraperitoneally administered one of the following cells into Balb/c mice: the parental DC2.4 cells, the DC2.4-CYP cells (DC2.4 cells transduced with lenti-CYP-GFP virus), and the DC-2.4-CYP-ALDH cells. Four days after the cell administration, mesenteric lymph nodes were examined by FACS (**Figure 4A**). Our data showed that the DC2.4-CYP-ALDH cells, when compared to the controls, significantly increased the abundance of foxp3$^+$CCR9$^+$ cells among CD3$^+$ T cells (**Figure 4B**,**C**). Based on these results, we conclude that the DC-CYP-ALDH cell administration significantly augments the induction of foxp3$^+$CCR9$^+$ T cells in peripheral lymphoid tissues.

FIGURE AND TABLE LEGENDS:

Figure 1: DC-CYP-ALDH cells expressed significantly increased amount of 1α-hydroxylase. (**A**) BMDC-CYP-ALDH cells were generated and analyzed by FACS. A representative FACS plot shows the expression of 1α-hydroxylase in the parental BMDCs and the BMDC-CYP-ALDH cells (gated on live cells). (**B**) 1α-hydroxylase substrate (i.e., 25(OH)D) was added into the DC cultures. Twenty-four h later, the supernatants were collected and 1,25$(OH)_2$D concentrations were measured. The data show concentrations of 1,25$(OH)_2$D in the cultures of the parental BMDCs, the BMDC-CYP cells, the BMDC-ALDH cells, and the BMDC-CYP-ALDH cells. **p < 0.01. ANOVA test. n = 4. This figure is adapted from Xu et al.[6]. Copyright 2019. The American Association of Immunologist, Inc.

Figure 2: DC-CYP-ALDH cells expressed significantly increased amount of RALDH2. (**A**) BMDC-CYP-ALDH cells were generated and analyzed as described in the protocol. Representative overlaid FACS plots show the BODIPY aminoacetate fluorescence in the BMDCs and the BMDC-CYP-ALDH cells in the presence (+DEAB) or absence (-DEAB) of the RALHD2 inhibitor diethylaminobenzaldehyde (DEAB). (**B**) Mean fluorescence intensities (MFIs) of BODIPY aminoacetate in the BMDCs and the BMDC-CYP-ALDH cells in the absence of DEAB. *p < 0.05; t-test; n = 4. This figure is adapted from Xu et al.[6]. Copyright 2019. The American Association of Immunologist, Inc.

Figure 3: DC-CYP-ALDH cells augmented the induction of foxp3$^+$CCR9$^+$ gut-homing Treg cells in vitro. (**A**) CD4$^+$ naive T cells were isolated from C57BL/6 mouse spleens. The CD4$^+$ T cells were then activated in cultures by an anti-CD3 mAb (5 μg/mL) in the presence of either the parental DC2.4 cells or the DC2.4-CYP-ALDH cells. Additionally, the cultures were added with the indicated

concentrations of 25(OH)D and retinol. Five days later, the cells were collected and analyzed by FACS for the expressions of foxp3 and CCR9 in $CD3^+CD4^+$ T cell population. Representative FACS plots show the expressions of foxp3 and CCR9 in the $CD3^+CD4^+$ T cell populations. (**B**) Cumulative data from (**A**). *$p < 0.05$; ANOVA test; n = 4. This figure is adapted from Xu et al.[6]. Copyright 2019. The American Association of Immunologist, Inc.

Figure 4: DC-CYP-ALDH cells augmented the induction of $foxp3^+CCR9^+$ gut-homing Treg cells in vivo. (**A**) Balb/c mice intraperitoneally (i.p.) received one of the following: DC transfers (Transfer), no DC transfer (No transfer), parental DC2.4 cells, DC2.4-CYP cells, and DC2.4-CYP-ALDH cells. Four days later, mesenteric lymph nodes (MLNs) were analyzed by FACS. (**B**) Representative FACS plots show the expressions of foxp3 and CCR9 in $CD3^+$ T cell population. (**C**) Cumulative data from (**B**) show the percentage of $foxp3^+CCR9^+$ cells in the $CD3^+$ T cell population. Cells were gated on $CD3^+$ T cells for all the analyses. Where applicable, the data presented are means ± SEM. *$p < 0.05$; ANOVA test; n = 4–6. This figure is adapted from Xu et al.[6]. Copyright 2019. The American Association of Immunologist, Inc.

DISCUSSION:

In this article we describe the use of DC-CYP-ALDH cells, for augmenting the induction of gut-homing Treg cells in peripheral lymphoid tissues. Our data have shown that the DC-CYP-ALDH cells can synthesize locally high concentrations de novo of both $1,25(OH)_2D$ and RA in vitro in the presence of corresponding substrates (i.e., 25(OH)D and retinol, respectively). Because sufficient blood concentrations of 25(OH)D and retinol can be easily achieved through vitamin D and A supplementations respectively in patients who have deficiencies[30,31], we reason that the DC-CYP-ALDH cells can augment the induction of gut-homing Treg cells in peripheral lymphoid tissues when normal blood concentrations of 25(OH)D and retinol are present. To support this reasoning, our data demonstrated that in normal healthy animals that do not have vitamin D and vitamin A deficiencies, the DC-CYP-ALDH cells augment the induction of Treg cells that express both regulatory molecules (i.e., foxp3 and IL-10) and a gut-homing receptor (i.e., CCR9). Therefore, this technology can be used for further investigation of gut-homing Treg cells for the treatment of IBD.

One critical step of this protocol is the production of lenti-CYP-ALDH virus with high titers. The preferred virus titers should be 10^8–10^9 TUs/mL. A high titer of the lenti-CYP-ALDH virus is necessary for a high transduction efficiency in DCs.

Another critical step of this protocol is the transduction efficiency in DCs. Because DC-CYP-ALDH cells are not tolerized in vitro in this technology, it is essential that the transduction rate be more than 90% to ensure that the DC-CYP-ALDH cells can efficiently augment the induction of gut-homing Treg cells in vivo. In addition, the DC-CYP-ALDH cells can be further purified by FACS before in vivo administration[32].

A unique advantage of this protocol is that the DC-CYP-ALDH cells do not need to be tolerized in vitro before in vivo administration. It is expected that the DC-CYP-ALDH cells, as a result of the combined actions of $1,25(OH)_2D$ and RA, will be maintained in a tolerogenic status in vivo because

both 1,25(OH)$_2$D and RA have been shown to tolerize DCs[33,34]. Therefore, we anticipate that the DC-CYP-ALDH cells will not have instability concerns in an in vivo proinflammatory environment.

Currently, we have only demonstrated that DC-CYP-ALDH cells can increase the frequency (number) of gut-homing Treg cells in peripheral lymphoid tissues and intestines[6]. Consequently, regulatory function in the intestines as a whole is enhanced because the percent of Treg cells in the intestines is increased. **Figure 4** shows that when compared to those with control treatments, intraperitoneal treatment with DC-CYP-ALDH cells significantly increased the percentage of CCR9$^+$foxp3$^+$ Treg cells in the mesenteric lymph nodes, meaning that more Treg cells in the mesenteric lymph nodes are able to specifically home into the intestinal tissues. **Figure 4** further shows that most foxp3$^+$ T cells in mesenteric lymph nodes are CCR9and therefore do not have gut-homing capacity. However, whether DC-CYP-ALDH cells can also enhance the regulatory function of each Treg cell per se (such as enhanced expression levels of foxp3 and/or IL-10) requires further investigation.

The reagents and materials described here are for animal studies only. However, the protocol is applicable for human studies by using corresponding human reagents and materials, except that DCs will be generated from peripheral blood monocytes in humans. The ultimate goal of this protocol is the generation of clinical grade DC-CYP-ALDH cells for the treatment of IBD.

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DISCLOSURES:

Drs. Xiaolei Tang and David J. Baylink are inventors of a pending patent related to this study.

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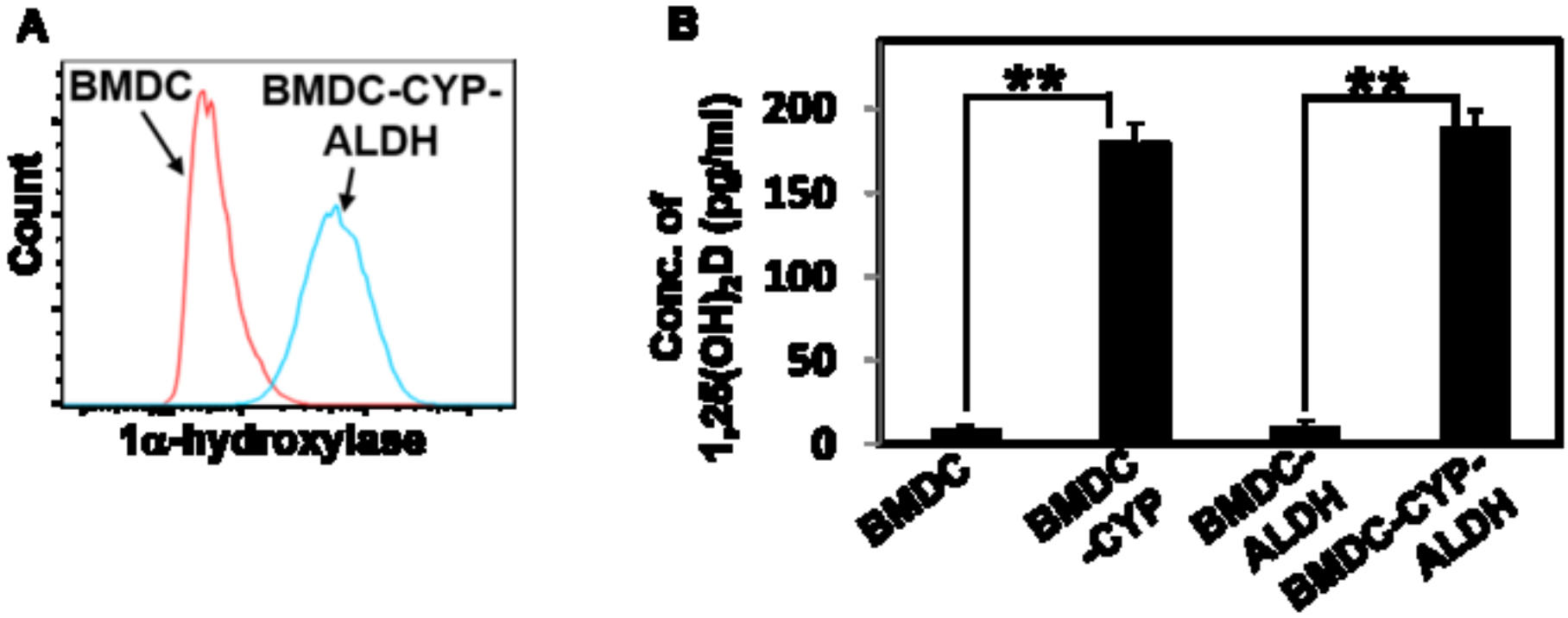

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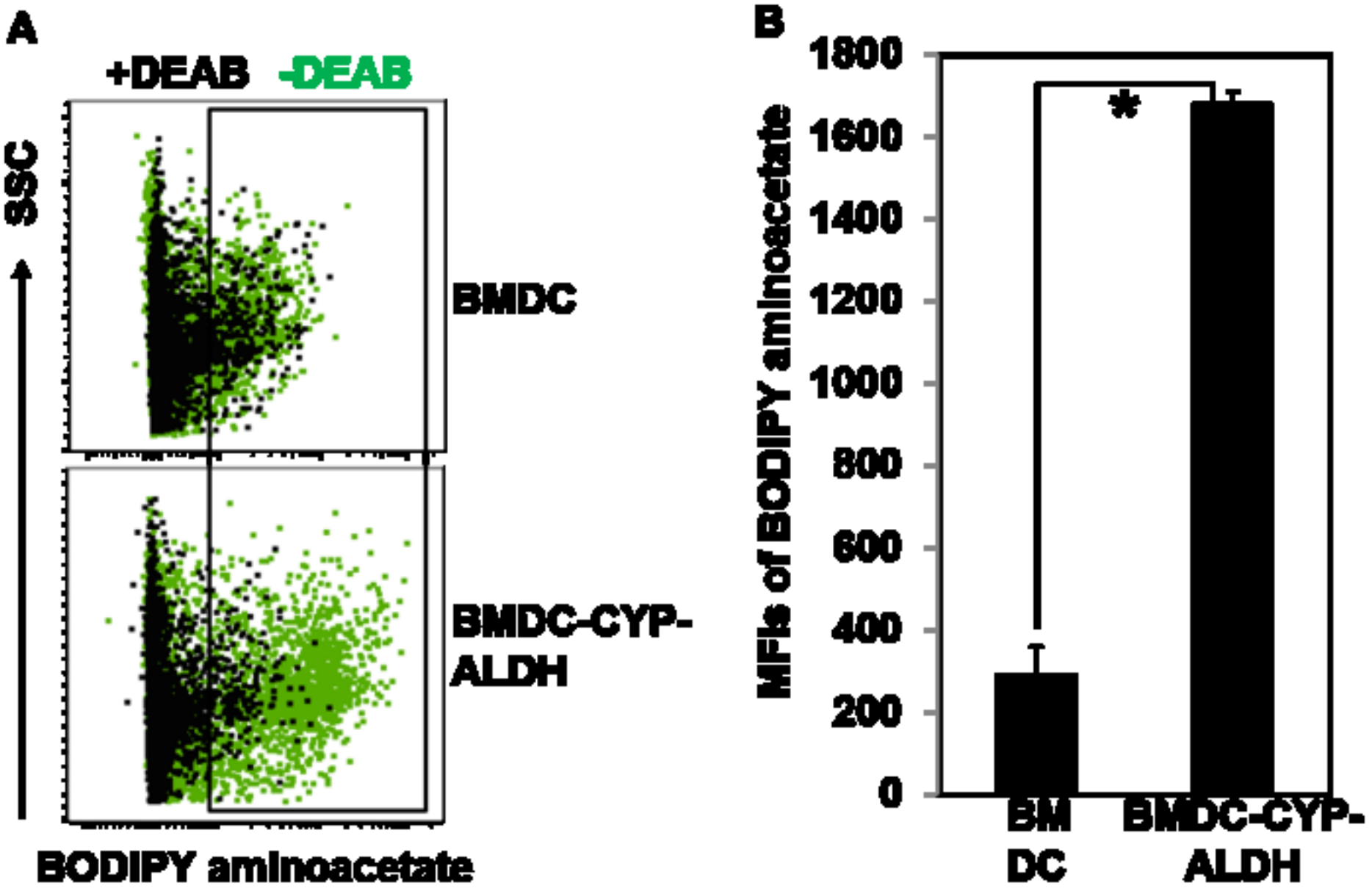

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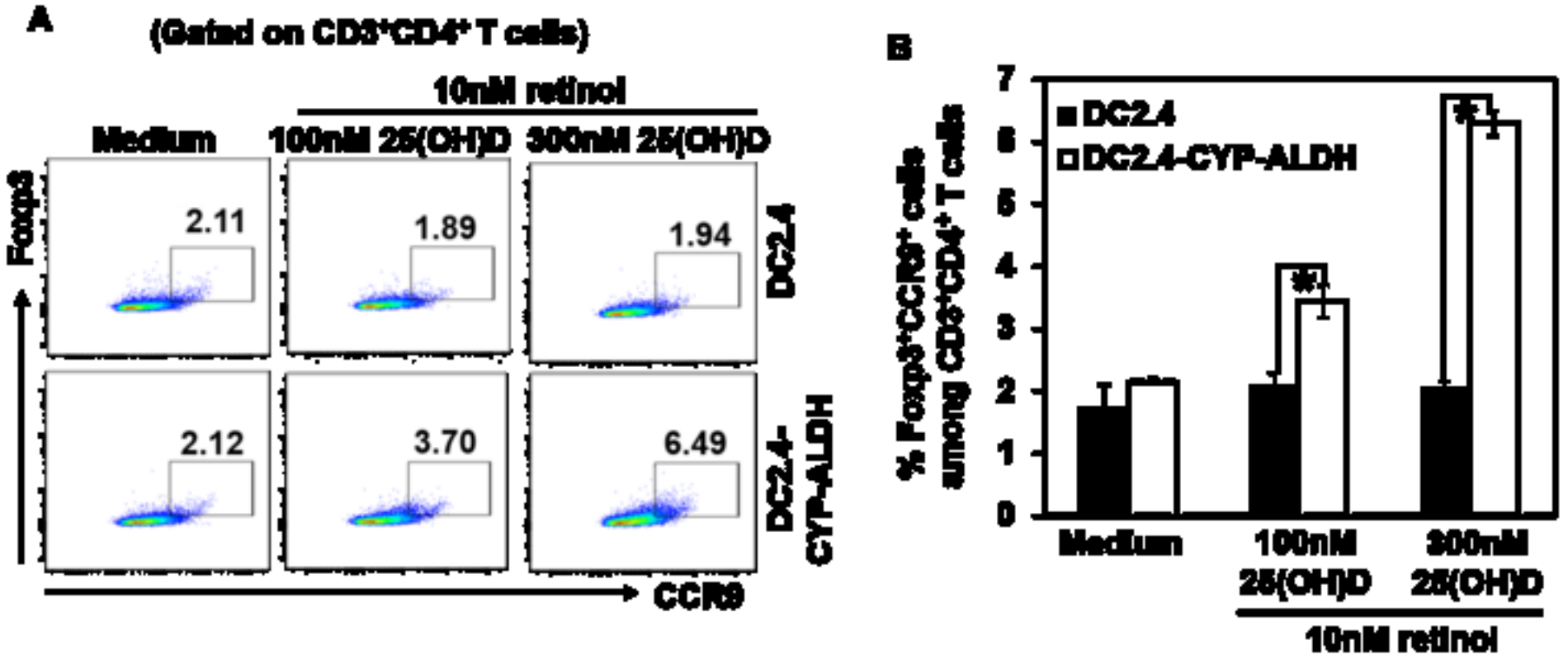

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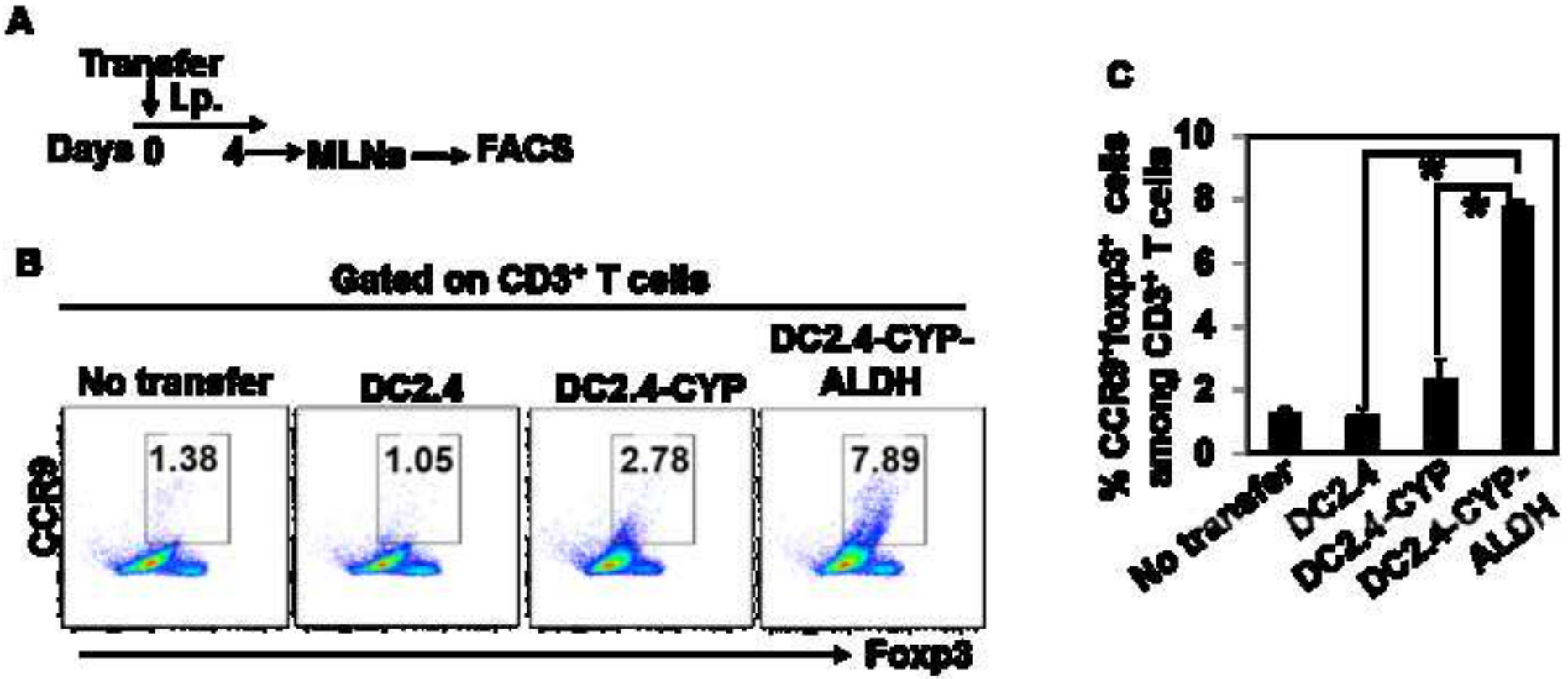

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Name of Material/ Equipment	Company
10 mL syringes	ThermoFisher Scientific
100 mm x 20 mm culture dishes	Sigma-Aldrich
12-well culture plates	ThermoFisher Scientific
150 mm x 25 mm culture dishes	Sigma-Aldrich
25-hydroxycholecalciferol (25[OH]D)	Sigma-Aldrich
293T cells	ATCC
2-mercaptoethanol	ThermoFisher Scientific
6-well culture plates	ThermoFisher Scientific
ALDEFLUOR kit	Stemcell Technologies
Anti-CYP27B1	Abcam
BD FACSAria II	BD Biosciences
CaCl2	Sigma-Aldrich
CM-10-D cell culture medium	
CM-10-R cell culture medium	
CM-4-D cell culture medium	
Corning bottle-top vacuum filters, 0.22 μM, 500 mL	Sigma-Aldrich
Corning bottle-top vacuum filters, 0.45 μM, 500 mL	Sigma-Aldrich
Dissecting scissor	ThermoFisher Scientific

DMEM medium	ThermoFisher Scientific
Fetal bovine serum	ThermoFisher Scientific
Forceps	ThermoFisher Scientific
Gibco ACK lysing buffer	ThermoFisher Scientific
Glycerol	Sigma-Aldrich
Goat anti-rabbit IgG	Abcam
HEPES	Millipore
Lenti-CYP-ALDH	Custom-made
L-glutamine	ThermoFisher Scientific
Lipopolysaccharide	Sigma-Aldrich
Murine GM-CSF	Peprotech
Murine IL-4	Peprotech
Na2HPO4	Sigma-Aldrich
NaCl	Sigma-Aldrich
Needles	ThermoFisher Scientific
Nonessential Amino Acids	ThermoFisher Scientific
pCMVR8.74	Addgene
Penicillin/Streptomycin	ThermoFisher Scientific
Phoshate Balanced Solution (PBS)	ThermoFisher Scientific
PMD2G	Addgene
Polypropylene tube, 15 mL	ThermoFisher Scientific
Polypropylene tube, 50 mL	ThermoFisher Scientific
Protamine sulfate	Sigma-Aldrich
Rabbit polycloncal IgG isotype control	Abcam

Radioimmunoassay for 1,25$(OH)_2$D measurement	Heartland Assays
RPMI 1640 medium, no glutamine	ThermoFisher Scientific
Sodium pyruvat	ThermoFisher Scientific
Sorvall Legend XTR Centrifuge	ThermoFisher Scientific
Sterile Cell strainers, 40 μm	ThermoFisher Scientific
Sterile storage bottles, 500 mL	ThermoFisher Scientific

Catalog Number
Cat# 03-377-23

Cat# CLS430167

Cat# 07-200-82
Cat# CLS430559
Cat# H4014
CRL-3216
Cat#: 21985023
Cat# 07-200-83
Cat# 01700
Cat# ab95047
N/A
Cat# C1016

Cat# CLS430513
Cat# CLS430514
Cat# 08-940

Cat# 11960044

Cat# 16000044
Cat# 22-327379
Cat# A1049201
Cat# G5516
Cat# ab205718
Cat# 391340
1.6-kb mouse CYP27B1 and ALDH1a2 cDNAs were amplified by PCR using a plasmid containing the CYP27B1 cDNA and a plasmid containing the ALDH1a2
Cat#25030081
Cat# L3755

Cat# 315-03

Cat# 214-14
Cat# NIST2186II
Cat# S9888
Cat# 14-841-02
Cat#: 11140076
Plasmid# 22036
Cat#15140148
Cat#: 20012027

Plasmid# 12259
Cat# AM12500
Cat# AM12502
Cat# P3369

Cat# ab171870

Cat# 21870076
Cat#: 11360070
Cat# 75004521
Cat# 07-201-430
Cat# CLS431432

Comments/Description

DMEM medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 0.055 μM 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, and 2 mM L-glutamine.
RPMI 1640 medium (no glutamine) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 0.055 μM 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, and 2 mM L-glutamine.
DMEM medium containing 4% fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 0.055 μM 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 0.1 mM nonessential amino acid, and 2 mM L-glutamine.

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20) Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Yes.

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22) As we are a methods journal, please ensure the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

A. ***Critical steps within the protocol.***
Yes.

B. Any modifications and troubleshooting of the technique.
Yes.

C. Any limitations of the technique.
Yes.

D. The significance with respect to existing methods.

Yes.

E. Any future applications of the technique.
Yes.

23) Please do not abbreviate the journal titles in the references section.
Yes.

24) Please sort the materials table in the alphabetical order.
Yes.

2. Reviewer 1:

1) Manuscript Summary: The manuscript by Bi at al describes a new method that can be used to induce CCR9+Foxp3+ Tregs using lentiviral in vitro transductions. The method is also used in a recent JI paper by authors.

2) Major Concerns:

A. It is unclear why author describe first the use of BMDCs but then switch to a DC-cell line. Please clarify and provide lentiviral transduction protocols for both, if different.
We used DC2.4 cells for mechanistic studies of DC-CYP-ALDH cells because DC2.4 cells are a cell line and are readily available. However, we used BMDCs for investigating the therapeutic effects of DC-CYP-ALDH cells for TNBS-induced colitis. The reason is that DC2.4 cells have a C57BL/6 background and the TNBS colitis is induced in Balb/c mice. The rationale for the choice of TNBS colitis is that it is mediated by Th1 cells and Th1 cells are recently shown to be critical in the pathogenesis of colitis.

The transduction protocol for both DC2.4 cells and BMDCs is the same.

B. It would be important to describe how the DC-identity of BM cells was or can be confirmed after the in vitro culture steps, ie what is the expect percentage of CD11c+ cells for example?
Normally, more than 90% of the DCs generated by our protocol are $CD11c^+$. DCs are routinely used in our studies[1,2].

C. In vivo experiments: How was the "gut-homing" of Tregs confirmed, now only the expression of CCR9 is determined in mesenteric lymphnode T cells. Did author confirm the gut homing by isolating the intra epithelial and/or lamina propria T regs? Were the proportions of CCR+ cells compared between mesenteric and inguinal/cervival lymph nodes? Were other gut homing markers for Tregs tested?
These information can be found in our JI paper[2]. Since this is a method paper, we are not including all the data published in our recent JI paper in this manuscript. We only requested the permission for reproducing the four figures presented in this paper.

To briefly address the reviewer's question, firstly, we confirmed the gut-homing by subcutaneous immunization with $OVA_{323-339}$-pulsed DC-CYP-ALDH cells followed by enumeration of I-A^d/$OVA_{323-339}$ tetramer$^+$ foxp3$^+$ and IL-10$^+$ Treg cells in intestines.

Secondly, we did not compare I-A^d/$OVA_{323-339}$ tetramer$^+$ foxp3$^+$ and IL-10$^+$ Treg cells between mesenteric and inguinal/cervical lymph nodes, which is an interesting experiment in the future to further define the specific homing capacity of the induced Treg cells.

Thirdly, other gut-homing receptors should be investigated as well in future studies.

D. How suppressive the Foxp3CCR9-positive Tregs are compared to CCR9-negative T regs? In Figure 4, it seems that CCR-negative Tregs tend to have have a higher expression of foxp3? Please include a discussion.

This is an interesting question. Currently, we have only demonstrated that DC-CYP-ALDH cells can increase the frequency (number) of gut-homing Treg cells in peripheral lymphoid tissues and intestines[2]. Consequently, regulatory function in intestines as a whole is enhanced because percent of Treg cells in intestines are increased. Figure 4 shows that intraperitoneal treatment with DC-CYP-ALDH cells, when compared to those with control treatments, significantly increased the percentage of CCR9$^+$foxp3$^+$ Treg cells in mesenteric lymph nodes, meaning that more Treg cells in the mesenteric lymph nodes are able to specifically home into intestinal tissues. Figure 4 further shows that most foxp3$^+$ T cells in mesenteric lymph nodes are CCR9$^-$ and therefore do not have gut-homing capacity. However, whether DC-CYP-ALDH cells can also enhance the regulatory function of each Treg cell per se (such as enhanced expression levels of foxp3 and/or IL-10) requires further investigation.

E. Experiment in Figure 2 is not sufficiently detailed. Please provide the a more detailed biochemical rationale in the method section. What are the DEAB+ and DEAB- cells is the facs blots, are they a mixture of two separate cell cultures or overlaid blots?

Sorry for the insufficient information that led to confusion in Figure 2. We have now provided detailed description of the protocol for the "Evaluation of the overexpressed RALDH2 in DC-CYP-ALDH cells" (section 4.2). "+DEAB" and "-DEAB" are overlaid two FACS plots in both BMDC and BMDC-CYP-ALDH panels, meaning that each panel contains FACS plots from two tubes in which one tube was added with DEAB and the other was not. "+DEAB" tubes are served as negative control for "-DEAB" tubes.

F. ***Did authors perform live gating in all their FACS analyses (FSC/SSC), if so please include this in the description and ideally show these blots, this is especially important for Figure 1 which now contains only a histogram.***

Yes. We routinely perform live gating for all FACS analyses. We now included this description in the figure legend.

3) Minor Concerns:

A. Please include a better description of thee custom made lenti-cyp-aldh plasmid, what is the backbone, is it commercially available?

We have now included more description of the custom made lenti-CYP-ALDH plasmid in "Materials". To address the reviewer's question, the backbone is Addgene's pRRL-SIN.cPPt.PGKGFP.WPRE lentiviral vector. The plasmid containing the CYP27B1 cDNA and the plasmid containing the ALDH1a2 cDNA are available from GeneCopoeia.

B. Can the generated lentivirus-containing supernatant infect humans, include necessary precautions if so.

All the reagents described in this protocol are for animal studies only and are not intended for human use.

3. Reviewer 2:

1) Manuscript Summary: This is a well written paper that covers an interesting and important topic building on the authors recent work in JI. It will be useful for researchers studying immune responses in the gut, regulatory T cell therapy or clinicians interested in IBD.

2) Major Concerns: The methods and presentation are sound and uncontroversial my concerns are

A. What does this add to the JI paper published earlier this year?

Our JI paper is more focused on describing the mechanisms of this technology. However, this JoVE paper will provide more detailed step-by-step description of the techniques that are used in the generation of DC-CYP-ALDH cells. Therefore, readers will find much easier to reproduce the technology by following the protocol described in this JoVE paper.

B. Could they look at using the same approach to generate gut homing Tregs in human cells? This should be straightforward and would extend the utility of the technique to those studying patient cells and those developing clinical grade Tregs. This should at least be covered in the discussion.

Yes, we are preparing for human studies. We have now added this discussion.

4. References:

1 Li, C. H. et al. Dendritic cells, engineered to overexpress 25-hydroxyvitamin D 1alpha-hydroxylase and pulsed with a myelin antigen, provide myelin-specific suppression of ongoing experimental allergic encephalomyelitis. FASEB J. doi:fj.201601243R [pii] 10.1096/fj.201601243R, (2017).

2 Xu, Y. et al. In Vivo Generation of Gut-Homing Regulatory T Cells for the Suppression of Colitis. J Immunol. 202 (12), 3447-3457, doi:10.4049/jimmunol.1800018 jimmunol.1800018 [pii], (2019).

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July 25, 2019

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