Chapter 3

Expansion of Regulatory T Cells In Vitro and In Vivo by IL-33

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Abstract

Thymic-derived, regulatory T cells (Treg) represent a subset of $CD4^+$ T cells that are required for normal immune homeostasis and suppression of unwanted responses against self-antigens (Ags) that prevent autoimmunity. Their role as immune regulators and potent ability to suppress T cell responses has been the focus of intense investigations aimed at utilizing these cells therapeutically, particularly in the settings of autoimmunity and transplantation. Many methods for expanding Treg have been described; however, efforts to generate large numbers of Treg for use in vivo often compromise their suppressor function or rely on the induction of Treg rather than their expansion. Our recent studies have focused on the barrier tissue-derived cytokine IL-33, a recently described IL-1 family member. IL-33 has emerged as a multifunctional protein, with reported roles in driving potent Type 1 and Type 2 immunity, as well as facilitating profound Treg expansion in vitro and in vivo. IL-33-expanded Treg express the IL-33 receptor (R) ST2, and express classical markers associated with Treg phenotype and suppressor function. They suppress both $CD4^+$ and $CD8^+$ T cell proliferation and effector functions in vitro, and Treg expressing ST2 have been identified as important regulators of detrimental immune responses in vivo. In the present chapter, we detail methods for expanding significant numbers of Treg using IL-33 both in vitro and in vivo that may potentially be used to promote/maintain organ transplant tolerance or suppress autoimmunity.

Key words Regulatory T cells, Interleukin-33, ST2, Dendritic cells, Transplantation tolerance, Interleukin-2

1 Introduction

The name Treg is a broadly used connotation that encompasses multiple subsets of functionally suppressive $CD4^+$ T cells, each with unique phenotypic and functional characteristics. The most widely characterized and studied are thymic-derived, $CD4^+$ T cells that undergo the T cell selection process and are released into the periphery. These cells express the transcription factor Forkhead box P 3 (Foxp3) and constitutively express high levels of the high-affinity IL-2R, CD25. A mutation in the Foxp3 gene in mice results in a disorder that closely resembles the fatal disorder IPEX (immune dysregulation, polyendocrinopathy, enteropathy, and

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X-linked inheritance) in humans and revealed a critical role for Foxp3 in the reported suppressive function of $CD4^+CD25^+$ regulatory T cells that control immune homeostasis and suppress autoimmunity [1–3]. Given their potent ability to suppress T cell responses, intense investigation has centered on harnessing these cells for therapeutic applications, particularly in the settings of transplantationand autoimmunity [4–6]. Many protocols have been described for expanding Treg, including polyclonal and Ag-specific cells [7]. Likewise, significant effort has been made to maintain or enhance their suppressor function, especially in the presence of inflammatory stimuli that could counter their regulatory mechanisms [8].

High constitutive expression of CD25 by Treg indicates that they rely heavily on IL-2 [9] and accordingly, culture of $CD4^+CD25^+$ T cells with IL-2 and TCR stimulation has been used as a method of Treg expansion in vitro [10]. Our recent published findings have revealed that IL-33, a pleiotropic cytokine with inflammatory and regulatory functions, stimulates IL-2 secretion by $CD11c^+$ DC (Fig. 1; [11]) that supports robust Treg expansion (Fig. 2; [11]). Additionally, IL-33 acting on DC in vitro results in selective expansion of IL-33R/$ST2^+$ Treg over $ST2^-$ Treg, as well as $Foxp3^-$ effector T cells (Fig. 2c; [11]). Administration of IL-33 in vivo also significantly expands Treg (Fig. 3; [11]) that suppress T cell responses (Fig. 4; [11]) and critically mediate fully MHC-mismatched experimental cardiac allograft survival [12, 13]. IL-33-expanded $ST2^+$ Treg express classical Treg phenotypic markers, and have recently emerged as key regulators of immune function [14]. Thus, the ability to selectively expand this unique Treg subset for potential therapeutic application is an important immunologic tool and is described in detail herein.

The observation that IL-33 expands Treg [11–13] was an important finding that added to the known pleiotropic functions

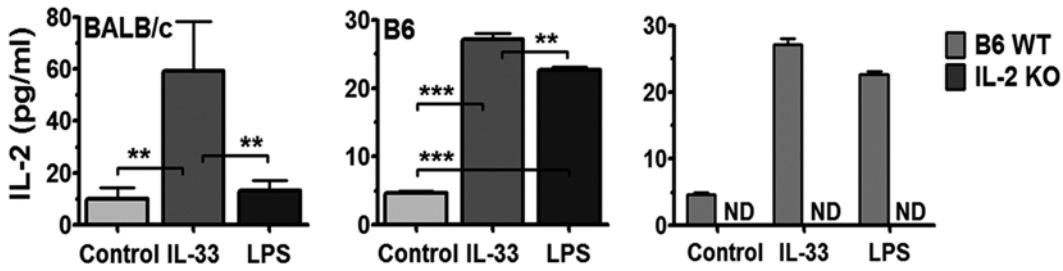

Fig. 1 IL-33 stimulates IL-2 secretion by $CD11c^+$ BMDCs. BALB/c, B6 wild-type (WT) or IL-$2^{-/-}$ bone marrow (BM)-derived $CD11c^+$ DC were generated in 7-day culture and cultured for 18 h in medium alone or supplemented with IL-33 (20 ng/ml) or LPS (100 ng/ml). Supernatants were harvested for cytokine quantitation by ELISA. Data are representative of $n=4$ (LPS) or $n=5$ (control and IL-33). ND = Not detectable, $^{**}p<0.01$, $^{***}p<0.001$. Figure and legend were modified and reproduced with permission from *The JournalofImmunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

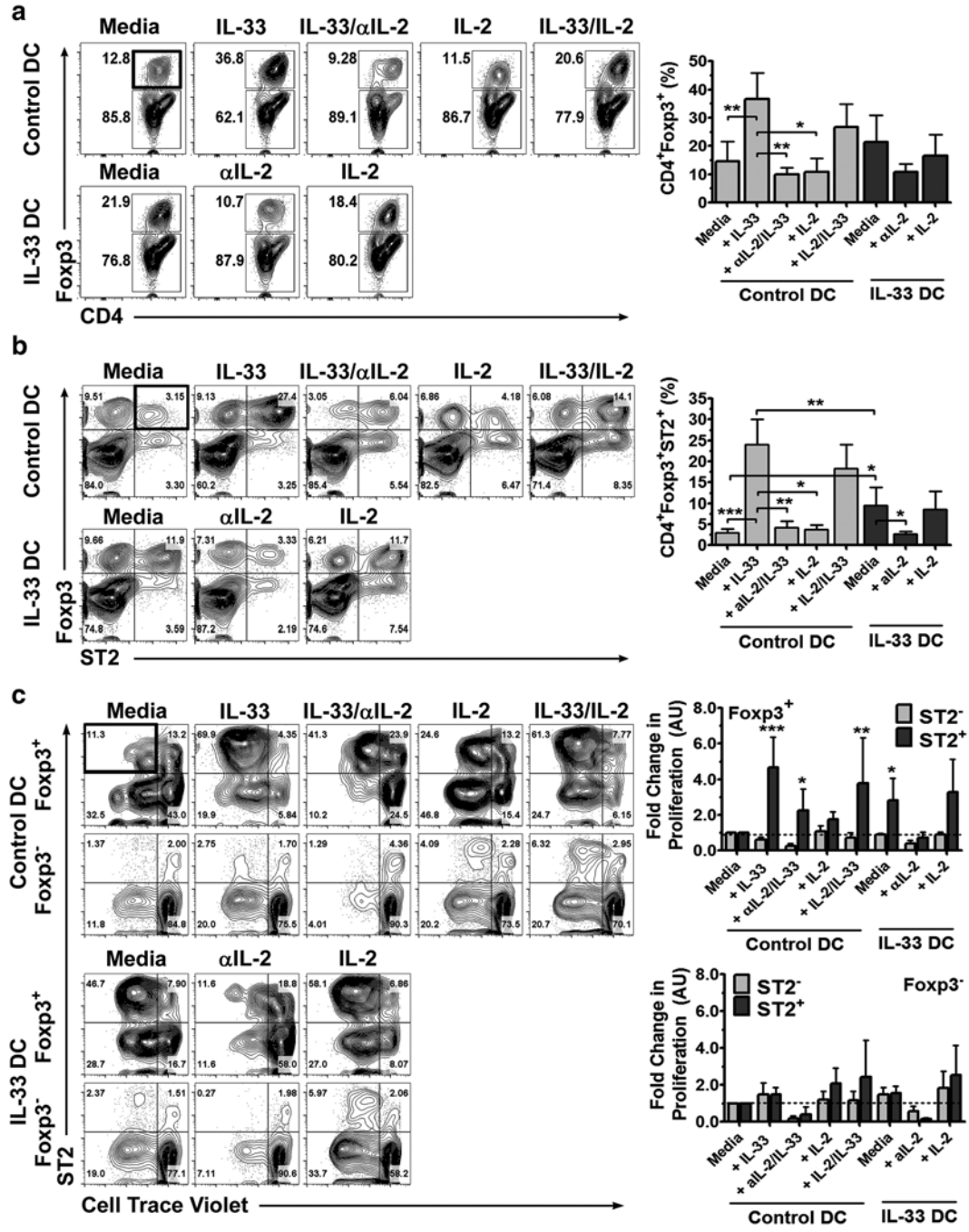

Fig. 2 CD11c⁺ BMDC-derived IL-2 promotes selective expansion of ST2⁺ Treg following IL-33 exposure. CD11c⁺ BALB/c BMDC were cultured overnight in media alone or supplemented with IL-33 (20 ng/ml). BMDC were cultured in MLR with Cell Trace Violet (CTV)-labeled B6 Foxp3-IRES-mRFP (FIR) CD4⁺ T cells for 5 days. Some wells were supplemented with IL-33 (10 ng/ml), neutralizing IL-2 antibody (10 μg/ml), rhIL-2 (50 U/ml), or a combination as indicated. After 5 days, cells were harvested and stained for flow cytometric analysis. Representative flow plots and an average of (**a**) Foxp3 and (**b**) Foxp3 and ST2 expression on CD4⁺-gated cells or (**c**) ST2 expression vs. CTV on CD4⁺ Foxp3⁺ (*top panels*) and CD4⁺Foxp3⁻ (*bottom panels*) cells. Results in were averaged from $n=4$ independent experiments. *Black boxes* on flow plots indicate populations used to generate corresponding graphs. *AU* Arbitrary Units for fold change reported in graphs in (**c**). *$p<0.05$, **$p<0.01$, ***$p<0.001$. Figure and legend were modified and reproduced with permission from *The Journal of Immunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

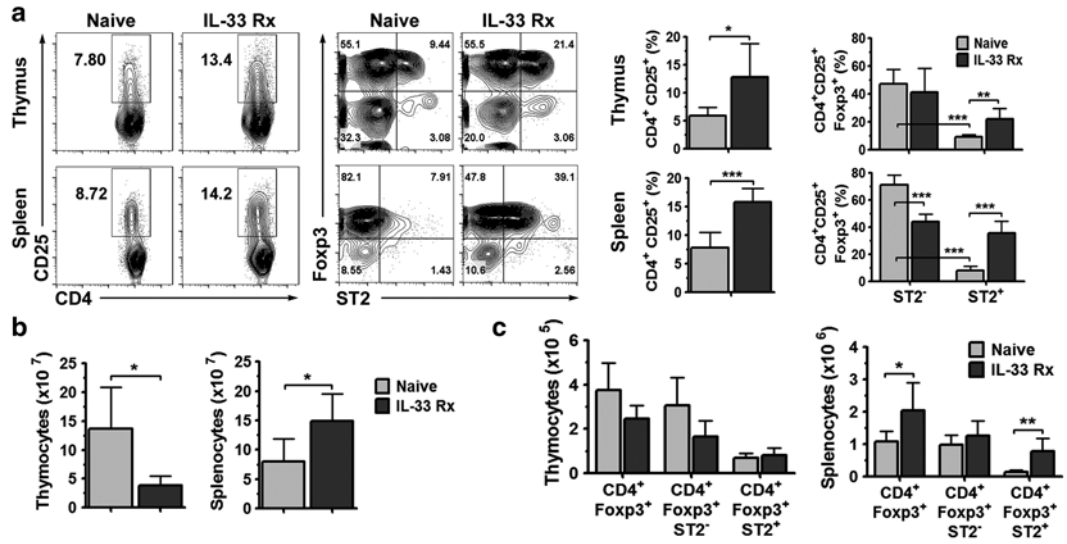

Fig. 3 IL-33 administration expandsan ST2$^+$ subset of CD4$^+$CD25$^+$Foxp3$^+$ T cells originating in the thymus. (**a**) Representative flow cytometry plots and frequency of CD4$^+$CD25$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ cells from CD3$^+$CD4$^+$-gated total thymocytes or splenocytes from naïve or IL-33-treated (IL-33 Rx) C57BL/6J (B6) mice. (**b**) Absolute number of total cells and (**c**) indicated cell populations in the thymus and spleens averaged from $n=5$ mice. $^*p<0.05$,$^{**}p<0.01$,$^{***}p<0.001$. Figure and legend reproduced with permission from *The Journal of Immunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

already ascribed to this IL-1 family cytokine [15]. IL-33 was initially found to promote potent Type 2 responses [16] through actions on ST2-expressing immune cells [17]. Through stimulation of IL-4, IL-5, and IL-13 production, these downstream effects of IL-33 are important in protective responses during parasitic infection [18], but conversely can severely exacerbate disease pathology associated with Type 2 responses [15]. It was later discovered that IL-33 could also act synergistically with proinflammatory stimuli, such as IL-12, to drive robust Type 1 immune responses in CD8$^+$ T cells [19], and NK and NKT cells [20], and stimulate potent antiviral immunity [21].

With the current use of Treg therapy already in clinical trials, studies continue in an effort to develop Treg expansion protocols tailored to specific uses. ST2$^+$ Treg may offer a therapeutic advantage over ST2$^-$ Treg in their ability to consume potentially detrimental IL-33 released from damaged tissues under pathologic conditions. We have demonstrated an ability for ST2$^+$ Treg to significantly suppress IL-12/IL-33-driven CD8$^+$ T cell IFN-γ production in vitro (Fig. 4; [11]) compared to ST2$^-$ Treg; however, this effect has not yet been reported in vivo. Given the pleiotropic effects of IL-33 in vivo, the capacity to selectively expand ST2$^+$ Treg ex vivo using IL-33-exposed CD11c$^+$ BMDC may be a promising way to capitalize on these important immunoregulatory cells. The following protocols describing robust expansion of ST2$^+$

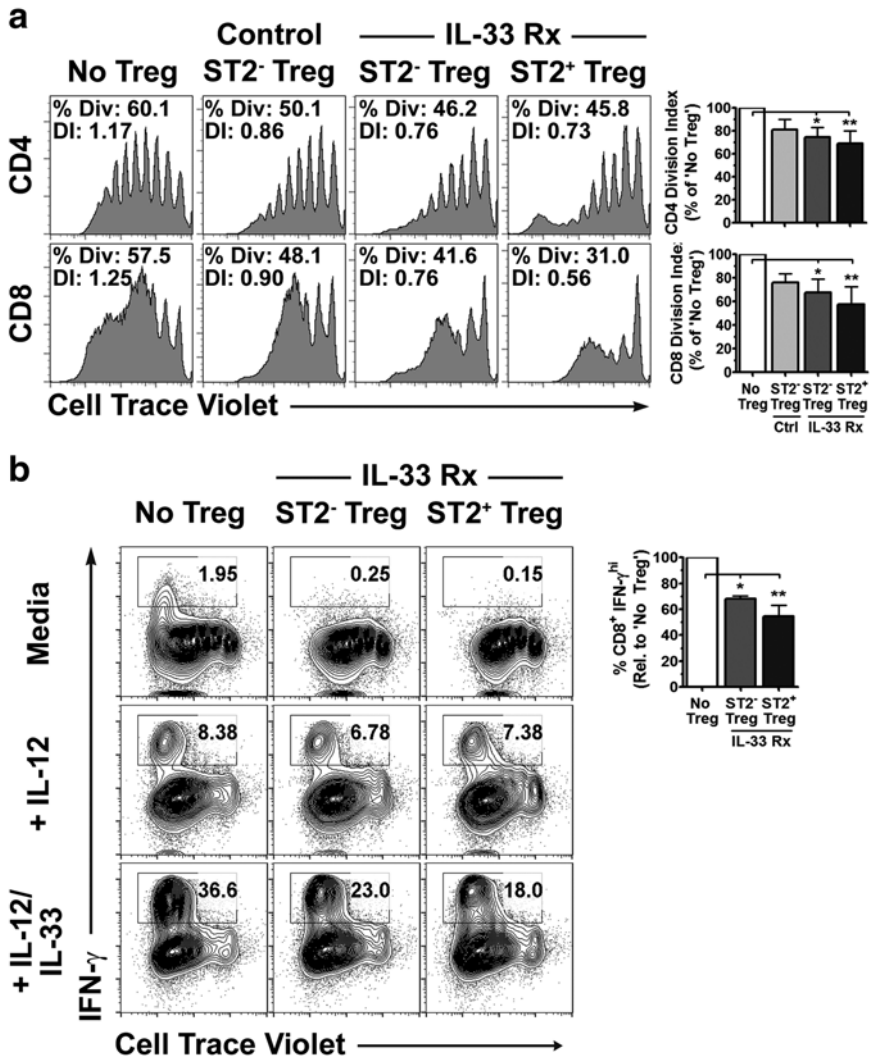

Fig. 4 IL-33-expanded Treg suppress CD8$^+$ effector T cell function. B6 FIR mice were administered IL-33 (0.5 μg/day; for 10 day). CD4$^+$ cells were purified on d 11 from total splenocytes and stained for flow sorting of CD4$^+$ RFP(Foxp3)$^+$ST2$^-$ or ST2$^+$ Treg. Sorted Treg were cultured with purified CTV-labeled, B6 CD4$^+$, or CD8$^+$ T cells at a Treg:Teffector ratio of 1:8 with CD3/CD28 T-activator beads for 4 days. Cultures were harvested and stained for flow cytometric analysis of T cell proliferation. (**a**) Representative flow plots and Division Index Relative to "No Treg" group of T cell proliferation based on CTV dilution profile. (**b**) Representative flow plots and an average of IFN-γ^{hi}CD8$^+$ T cells unstimulated or stimulated with 5 ng/ml IL-12 alone or in combination with 10 ng/ml IL-33. Data were generated from $n=3$ independent experiments. Statistical significance in A and B were determined by one-way ANOVA. DI = Division Index. *$p<0.05$, **$p<0.01$. Figure and legend reproduced with permission from *The JournalofImmunology* [11]. (Copyright 2014. The American Association of Immunologists, Inc.)

Treg using IL-33 in vitro and in vivo provide the avenue for further exploration, including investigation of ST2$^+$ Treg in humans, of the function and potential therapeutic use of this unique Treg subset.

2 Materials

2.1 Administration of Recombinant Mouse IL-33 and Spleen Harvest

1. Phosphate-buffered saline (PBS; Lonza, Walkersville, MD).
2. Recombinant mouse IL-33 (BioLegend, San Diego, CA) is resuspended in PBS at a final concentration of 5–10 μg/ml. IL-33 is administered by intraperitoneal (i.p.) injection at 100 μl/mouse (0.5–1 μg/mouse) daily for 10 days.
3. Fetal bovine serum (FBS)/EDTA solution: 500 ml PBS, 0.5 % heat-inactivated FBS, 2 mM EDTA (Sigma, St. Louis, MO).
4. Complete RPMI 1640 media: RPMI 1640 (Gibco by Life Technologies, Grand Island, NY) supplemented with 10 % heat-inactivated FBS, 50 μM 2-mercaptoethanol, 2 mM l-glutamine, 1 M MEM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid).
5. Red blood cell (RBC) lysis buffer: 1 L MilliQ water, 0.1 mM EDTA, 1 g potassium bicarbonate ($KHCO_3$), 8.29 g ammonium chloride (NH_4Cl). Adjust pH to 7.2–7.5 and filter.
6. Sterile needles, 27 gauge, 1/2 in.
7. Sterile syringes (1 and 5 cc).
8. Nylon mesh cell strainers, 70 μm (BD Biosciences, Franklin Lakes, NJ).
9. Sterile Petri dish, 100 mm × 15 mm (Fisher Scientific, Pittsburgh, PA).

2.2 Expansion of Treg In Vitro

2.2.1 Propagation of $CD11c^+$ Cells from Mouse Bone Marrow

1. Recombinant murine IL-4 (R&D Systems, Minneapolis, MN).
2. Recombinant murine GM-CSF (R&D Systems, Minneapolis, MN).
3. Mouse Anti-CD11c immunomagnetic beads (Miltenyi Biotec, Auburn, CA).
4. MACS® Separation Columns (Miltenyi Biotec, Auburn, CA).
5. MACS® Separator Magnet (Miltenyi Biotec, Auburn, CA).
6. Bright-Line™ Hemacytometer (Fisher Scientific, Pittsburgh, PA).
7. Cell Culture Dish, 100 mm tissue culture-treated polystyrene (BD Biosciences).
8. 0.4 % (w/v) trypan blue in 0.81 % sodium chloride and 0.06 % potassium phosphate, dibasic (Sigma-Aldrich, St. Louis, MO).
9. Needles, syringes, cell strainers, PBS, FBS/EDTA, and RPMI 1640 medium—*see* Subheading 2.1.

2.2.2 Expansion of Treg In Vitro by IL-33 and CD11c+ BMDC

1. *See* Subheading 2.1 for necessary materials for isolation of total splenocytes.
2. T1/ST2 (IL-33R) FITC-conjugated mAb (MD Bioproducts, St. Paul, MN), Anti-mouse CD4 (Clone RM 4-5) APC-conjugated mAb (Affymetrix/eBioscience, San Diego, CA).
3. 5 % FBS: 500 ml PBS, 25 ml heat-inactivated FBS.
4. Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies).
5. DynaMag™-15 Magnet (Life Technologies).
6. Recombinant mouse IL-33 (BioLegend).

2.3 Flow Cytometric Analysis of IL-33-Expanded Treg

1. 5 ml Round-bottom polystyrene tubes (BD Falcon).
2. Cell stain buffer (CSB): 500 ml PBS, 5 % heat-activated FBS, 0.1 % (w/v) sodium azide, pH 7.4–7.6.

2.3.1 Surface Staining

3. 5 % Normal goat serum (GS) in CSB.
4. Appropriate monoclonal antibodies (mAbs).
5. 4 % Paraformaldehyde (PFA): 4 g of PFA per 100 ml of PBS. Can be diluted to 1–2 % working solution with PBS to fix cells.

2.3.2 Intracellular Staining

1. Permeabilization buffer (PB): 500 ml PBS, 5 % heat-inactivated FBS, 0.1 % sodium azide, 0.1 % saponin.
2. Fixation/permeabilization buffer (Fix/Perm): Foxp3/Transcription Factor Staining Set (Affymetrix/eBioscience).
3. 5 % Normal goat serum in PB.
4. Appropriate mAbs.
5. 4 % Paraformaldehyde (PFA): 40 g PFA in 1 L PBS. Store at 4 °C protected from light.

3 Methods

3.1 Expansion of Treg In Vivo by Administration of Recombinant Mouse IL-33

1. Dissolve recombinant mouse IL-33 in PBS to a final concentration of 5–10 μg/ml.
2. Using a 1 cc syringe with a 27G, ½″ needle, administer 100 μl by intraperitoneal (i.p.) injection once daily for 10 days to C57BL/6 (B6) mice. Foxp3 reporter mice may be used to permit flow sorting of expanded Treg for functional assays (Foxp3-IRES-mRFP (FIR) on a C57BL/6 background) following IL-33 administration.
3. One day after the final injection, sacrifice animals using approved methods of euthanasia. Harvest the spleen using aseptic techniques and place in FBS/EDTA on ice.

4. Transfer the spleen to a sterile Petri dish and mechanically dissect into small pieces no larger than 2–3 mm. The dissected spleen should be forced through a 70 μm nylon mesh cell strainer into a 50 ml conical tube using a sterile 5 ml syringe. Wash the dish and cell strainer with 20–30 ml cold FBS/EDTA.
5. Centrifuge at 500 × *g* for 5 min at 4 °C, and resuspend the cells in RBC lysis buffer (3–5 ml per spleen) for 5 min.
6. Following lysis, add PBS to bring the total volume to 50 ml, and centrifuge the cells again under the same conditions. Splenocytes are ready for purification or immunostaining for flow sorting or phenotypic analysis. Keep cells in RPMI 1640 medium on ice until ready to use.

3.2 Expansion of Treg In Vitro Using IL-33 and CD11c+ Dendritic Cells

3.2.1 Generation of CD11c+ BMDC

1. From 8- to 12-week-old mice, dissect the femurs and tibiae, taking care to remove as much muscle as possible, and place in a 50 ml tube with FBS/EDTA on ice.
2. Under sterile conditions in a laminar flow hood, place the bones in a sterile Petri dish with 10–15 ml FBS/EDTA. Sterile scissors and forceps are used to tease away and remove any remaining muscle tissue. Place cleaned bones ina second Petri dish with 10–15 ml fresh FBS/EDTA.
3. Using scissors, carefully cut the bones and flush the BM into a clean dish with FBS/EDTA using a 5–10 cc syringe and 27G needle.
4. Once all the bones have been flushed, remove the needle and use the syringe to transfer the cells to a 50 ml tube, passing them through a 70 μm nylon cell strainer. Wash the dish and strainer with 5–10 ml FBS/EDTA.
5. Centrifuge at 500 × *g* for 5 min at 4 °C.
6. Carefully aspirate the supernatant, taking care not to disrupt the pellet. Resuspend the cells in 3–5 ml RBC lysis buffer for 5 min.
7. Add cold FBS/EDTA to 50 ml and centrifuge as described above.
8. Resuspend the cells in RPMI 1640 and count viable cells using Trypan Blue and a hemacytometer.
9. Culture cells in a sterile, 100 mm tissue culture-treated dish at $3–4 \times 10^6$ cells in 15 ml RPMI 1640 supplemented with GM-CSF and IL-4 at 1000 U/ml (day 0). Cells will be cultured for a total of 7 days.
10. On day 2, remove 10 ml of tissue culture media per dish and centrifuge at 500 × *g* for 5 min at 4 °C. Pour off the supernatant and resuspend cells in 15 ml fresh RPMI 1640 per dish with GM-CSF and IL-4.

11. On day 4, gently swirl the plates and remove 15 ml media containing floating cells. Discard this media and add 15 ml fresh RPMI 1640 per dish with GM-CSF and IL-4.
12. On day 6, repeat process for day 2, removing 15 ml, spinning down cells, pouring off the supernatant and resuspending cells in 15 ml fresh RPMI per dish with GM-CSF and IL-4.
13. On day 7, gently swirl plates and harvest all media/non-adherent cells into a 50 ml tube. Add 5 ml FBS/EDTA per dish and pipette up and down to wash thoroughly. Collect these cells and add to 50 ml tube.
14. Centrifuge at 500 × *g* for 5 min at 4 °C.
15. Resuspend cells in FBS/EDTA and count using hemacytometer. Proceed with CD11c isolation using mouse anti-CD11c immunomagnetic beads (Miltenyi Biotec) according to the manufacturer's protocol.
16. Resuspend positively-selected CD11c$^+$ cells in RPMI 1640 medium and count. Bring cells to a concentration of 1×10^5 cells/ml and keep on ice. Cells are ready for culture.

3.2.2 Expansion of Treg In Vitro by CD11c$^+$ BMDC and IL-33

1. Prepare total splenocyte single-cell suspension from untreated/naïve wild-type (WT) B6 or FIR B6 mice as described in Subheading 3.1.
2. Following RBC lysis, isolate untouched bulk CD4$^+$ T cells by negative selection using Dynabeads® Untouched™ Mouse CD4 Cells Kit (Life Technologies) according to the manufacturer's instructions.
3. Remove non-CD4$^+$T cells using the DynaMag™-15 Magnet (Life Technologies).
4. Wash the isolated CD4$^+$ T cells in FBS/EDTA. Centrifuge at 500 × *g* for 5 min at 4 °C.
5. Pour off the supernatant and resuspend cells in RPMI 1640 medium. Count cells using trypan blue and a hemacytometer.
6. Dilute CD4$^+$ T cells to a concentration of 1×10^6 cells/ml in RPMI 1640 medium.
7. In a 96-well U-bottom plate, add purified CD11c$^+$ BMDC (100 µl from Subheading 3.2.1) and bulk CD4$^+$ T cells at a DC:T cell ratio of 1:10 in 200 µl total volume (*see* **Note 1**). In a syngeneic system when B6 CD11c$^+$ BMDC are used with B6 FIR CD4$^+$ T cells, anti-CD3 is added at a final concentration of 0.25 µg/ml (*see* **Note 2**).
8. Recombinant IL-33 is added to each well at a final concentration of 10 ng/ml (*see* **Notes 3** & **4**).
7. Cells are cultured for a total of 5 days at 37 °C, in a humidified 5 % CO_2 incubator.

8. On day 5, cells are harvested by pipetting up and down and transferred to a 50 ml tube.
9. Centrifuge at 500 × *g* for 5 min at 4 °C.
10. The cells are ready for immunostaining for flow sorting or phenotypic analysis.

3.3 Flow Sorting of IL-33-Expanded Treg for Functional Assays

3.3.1 Immunostaining and FlowSorting of IL-33-Expanded Treg

1. Utilizing Foxp3-IRES-mRFP (FIR) reporter mice, Treg expanded by IL-33 in vivo (Subheading 3.1) or in vitro (Subheading 3.2) may be flow-sorted for testing of their functional suppressive capacity, and comparison of IL-33R/ST2$^+$ vs. ST2$^-$Foxp3$^+$ Treg subsets.
2. Total splenocytes from FIR mice (Subheading 3.1) or cells harvested 4 °C from culture (Subheading 3.2) are resuspended in 5 % FBS and stained using ST2-FITC (1:200 dilution) and CD4-APC (1:800 dilution). Cells are incubated at 4 °C for 30 min.
3. Wash cells with 10× volume 5 % FBS and centrifuge at 500 × *g* for 5 min at 4 °C.
4. Resuspend cells in 5 % FBS and filter through cell strainer cap into 5 ml round bottom tube.
5. Cells are sorted using a FACSAria™ (BD Biosciences). Foxp3$^+$ (RFP$^+$) cells are detected using the PE/Texas Red channel. Gating on lymphocytes (FSC-A vs. SSC-A) and single cells (FSC-H vs. FSC-W), ST2$^+$ and ST2$^-$Foxp3$^+$ cells are sorted from the CD4$^+$-gated population. Sorted cells are collected in sterile 5 ml snap-cap round-bottom tubes containing approximately 1 ml cold RPMI 1640 medium.
6. Sorted cells are washed in RPMI 1640 and centrifuged at 500 × *g* for 5 min at 4 °C.
7. Cells are resuspended in RPMI 1640 and counted using trypan blue and a hemacytometer. Cells are ready for in vitro suppressionassay or phenotype analysis by flow cytometry. For in vivo use, wash cells with PBS to remove traces of FBS.

3.3.2 Testing the T Cell Suppressive Capacity of IL-33-Expanded Treg In Vitro

1. The T cell suppressor function of flow-sorted Treg populations (Subheading 3.3.1) can be tested in vitro. CD4$^+$CD25$^-$ T effector (T_{eff}) cells are used as responders and purified from total splenocytes of a naïve B6 mouse. To do this, purified rat anti-mouse CD25 (clone PC61 at 1:100) is included in the antibody cocktail during the CD4$^+$ T cell purification process (described in Subheading 3.1).
2. Purified CD4$^+$CD25$^-$ T cells are labeled with CellTrace™ Violet (CTV) Cell Proliferation kit (Life Technologies) according to the manufacturer's instructions, taking care to keep cells in the dark during and after the labeling process.

3. Resuspend CTV-labeled cells in RPMI 1640 medium and count. Adjust the concentration to 1×10^6/ml.
4. CTV-labeled T_{eff} cells (1×10^5/well) are stimulated in 96-well U-bottom plates with Dynabeads® Mouse T-Activator CD3/CD28 (1×10^4/well; Life Technologies). Flow-sorted Treg populations are added at different Treg:T_{eff} ratios in a final volume of 200 μl per well and cells are cultured for 3 days at 37 °C, in a humidified 5 % CO_2 incubator.
5. At the end of 3 days, cells are harvested from culture and stained for flow cytometric quantification of T_{eff} cell proliferation by CTV-dilution analysis. Flow-sorted $CD4^+$ Treg can be distinguished from $CD4^+$ T_{eff} by staining for CD90.1/Thy1.1 and exclusion of $CD90.1^+$ cells from final analysis.
6. Cells are acquired using an LSRFortessa (BD Biosciences) and data is analyzed using FlowJo software (v10; Tree Star, Ashland, OR).

3.4 Phenotypic Analysis of IL-33-Expanded Treg by Flow Cytometry

1. Surface Ag expression can be analyzed by multicolor flow cytometry on total splenocytes or CD4-purified cells following 10 day administration of IL-33 or cells harvested from DC:T cell co-culture following in vitro expansion.

3.4.1 Surface Staining

2. Specifically, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, allophycocyanin (APC)/AlexaFluor647-, Pacific Blue-, PerCP-Cy5.5-, and PE-Cy7-conjugated antibodies are used to stain Treg.
3. Typically, 5×10^5 cells are blocked with 5 % normal goat serum (GS) in Cell Stain Buffer (CSB) (10 min; 4 °C), then stained with appropriate mAbs (30 min; 4 °C) in CSB. Appropriate isotype-matched IgGs should be included as negative controls.
4. The mAbs (clones) used for Treg surface staining are: CD3 (17A2), CD4 (RM 4-5), CD25 (PC61), ST2 (DJ8), CD304/Neuropilin-1 (3E12), CD44 (IM7), and CD278/ICOS (7E.17G9).
5. Wash cells twice in CSB. Data should be acquired immediately if using Foxp3 (FIR) reporter mice (*see* **Note 5**).
6. Data are acquired with a LSRFortessa flow cytometer (BD ImmunoCytometry Systems; San Jose, CA) and analyzed using FlowJo software (v10; Tree Star).

3.4.2 Intracellular Staining

1. To determine expression of transcription factors (from cells stained in 3.4.1 generated using WT mice and not FIR reporter mice), perform intracellular staining immediately following surface staining. The mAbs (clones) used for intracellular staining include: Foxp3 (FJK-16 s), GATA-3 (16E10A23), T-bet (4B10), and Helios (22F6).

2. After the second wash with CSB, resuspend cells in 100 µl Fix/Perm buffer and immediately vortex. Incubate at room temperature (RT) for 45 min, or alternatively, up to 16 h at 4 °C.
3. After the incubation in Fix/Perm buffer, wash the cells with 400 µl permeabilization buffer (PB) and centrifuge at 500 × *g* for 5 min at 4 °C. Aspirate the supernatant and resuspend in PB + 3 % GS and desired mAbs. Incubate for 30 min at 4 °C.
4. Wash the cells (2×) with PB.
5. Resuspend cells in CSB and acquire, of fix with 1–2 % paraformaldehyde (PFA).

4 Notes

1. For optimal yield of ST2$^+$Foxp3$^+$ Treg, bulk CD4$^+$ T cells should be used as the starting population during in vitro expansion with CD11c$^+$ BMDC and IL-33. Although they still undergo expansion, we have found that the yield of ST2$^+$ Treg is actually lower when starting with purified CD4$^+$CD25$^+$ T cells.
2. For applications where MHC-mismatch is a factor, in vitro expansion of Treg by IL-33 can be achieved using allogeneic (BALB/c) CD11c$^+$ BMDC in place of syngeneic (B6) CD11c$^+$ BMDC + anti-CD3, with comparable results.
3. ST2$^+$ Treg can be generated in vitro in 5 day culture with CD11c$^+$ BMDC that were exposed to 20 ng/ml IL-33 for 18 h, without the addition of exogenous IL-33 (Fig. 2; [11]). The overall yield of Treg may be lower compared to cultures with IL-33 directly added to BMDC:T cell co-culture; however, the resulting phenotype and suppressor function of Treg generated by either method are comparable.
4. Recombinant IL-2 is not required during in vitro Treg expansion using CD11c$^+$ BMDC and IL-33. We have reported that adding exogenous IL-2 does not augment Treg expansion above levels achieved with CD11c$^+$ BMDC and IL-33 (Fig. 2; [11]), and IL-33 stimulates IL-2 secretion by DC to support Treg expansion (Fig. 1; [11]).
5. For phenotypic analysis of surface markers only on Treg, FIR reporter mice can be used to identify Treg to avoid intracellular staining (identification of Foxp3$^+$/RFP$^+$ cells during flow acquisition). Cells must be acquired immediately following staining, since fixation with PFA will quench RFP fluorescence. For analysis of intracellular proteins, including cytokines and transcription factors, WT mice are used with intracellular Foxp3 staining.

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References

1. Bennett CL, Christie J, Ramsdell F et al (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 27(1):20–21
2. Wildin RS, Ramsdell F, Peake J et al (2001) X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet 27(1):18–20
3. Fontenot JD, Gavin MA, Rudensky AY (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4(4):330–336
4. McMurchy AN, Bushell A, Levings MK, Wood KJ (2011) Moving to tolerance: clinical application of T regulatory cells. Semin Immunol 23(4):304–313
5. Tang Q, Bluestone JA, Kang SM (2012) CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. J Mol Cell Biol 4(1):11–21
6. Verbsky JW (2007) Therapeutic use of T regulatory cells. Curr Opin Rheumatol 19(3): 252–258
7. Shevach EM (2011) Biological functions of regulatory T cells. Adv Immunol 112:137–176
8. Campbell DJ, Koch MA (2011) Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nat Rev Immunol 11(2):119–130
9. Almeida AR, Legrand N, Papiernik M, Freitas AA (2002) Homeostasis of peripheral CD4+ T cells: IL-2R alpha and IL-2 shape a population of regulatory cells that controls CD4+ T cell numbers. J Immunol 169(9):4850–4860
10. Hoffmann P, Eder R, Kunz-Schughart LA et al (2004) Large-scale in vitro expansion of polyclonal human CD4(+)CD25high regulatory T cells. Blood 104(3):895–903
11. Matta BM, Lott JM, Mathews LR et al (2014) IL-33 is an unconventional alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells. J Immunol 193(8):4010–4020
12. Turnquist HR, Zhao Z, Rosborough BR et al (2011) IL-33 expands suppressive CD11b+ Gr-1(int) and regulatory T cells, including ST2L+ Foxp3+ cells, and mediates regulatory T cell-dependent promotion of cardiac allograft survival. J Immunol 187(9):4598–4610
13. Brunner SM, Schiechl G, Falk W et al (2011) Interleukin-33 prolongs allograft survival during chronic cardiac rejection. Transpl Int 24(10):1027–1039
14. Schiering C, Krausgruber T, Chomka A et al (2014) The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature 513(7519):564–568
15. Liew FY, Pitman NI, McInnes IB (2010) Disease-associated functions of IL-33: the new kid in the IL-1 family. Nat Rev Immunol 10(2):103–110
16. Schmitz J, Owyang A, Oldham E et al (2005) IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 23(5):479–490
17. Liu Q, Turnquist HR (2013) Implications for interleukin-33 in solid organ transplantation. Cytokine 62(2):183–194
18. Humphreys NE, Xu D, Hepworth MR et al (2008) IL-33, a potent inducer of adaptive immunity to intestinal nematodes. J Immunol 180(4):2443–2449
19. Yang Q, Li G, Zhu Y et al (2011) IL-33 synergizes with TCR and IL-12 signaling to promote the effector function of CD8+ T cells. Eur J Immunol 41(11):3351–3360
20. Bourgeois E, Van LP, Samson M et al (2009) The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. Eur J Immunol 39(4):1046–1055
21. Bonilla WV, Frohlich A, Senn K et al (2012) The alarmin interleukin-33 drives protective antiviral CD8(+) T cell responses. Science 335(6071):984–989