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Reconstitution of Lymphopaenic Mice with Regulatory and Conventional T Cell Subsets

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[Abstract] Transfer of mature T cells into immunodeficient mice results in sub-optimal reconstitution of the peripheral T cell pool. Under lymphopenic conditions, dendritic cells are released from tonic control by regulatory T cells (Tregs), and consequently drive activation and proliferation of low affinity T cells specific for endogenous antigens. This oligoclonal proliferation results in a T cell population dominated by T cells possessing an effector/memory phenotype and a limited TCR repertoire. Oligoclonal expansion can be prevented by selectively reconstituting the Treg compartment prior to T cell transfer (Bolton *et al*., 2015). Reconstitution of the Treg compartment of lymphopenic mice has been tested in immunodeficient mouse strains such as *Rag-1*$^{-/-}$or *Rag-2*$^{-/-}$ mice, and in immunosufficient mice rendered transiently lymphopaenic by lethal whole body irradiation as conditioning for bone marrow transplantation (BMT). Transfer of purified Tregs into these hosts, combined with treatment with exogenous IL-2 for 7 days, is sufficient to reconstitute the Treg compartment and reduce expression of dendritic cell costimulatory molecules, a critical process in preventing inappropriate expansion of self-reactive T cells. T cells transferred after Treg reconstitution do not undergo rapid spontaneous proliferation, and instead undergo slow homeostatic division to repopulate the T cell pool with naive T cells, thus allowing optimal reconstitution of peripheral T cell pool.

Materials and Reagents

1. 50 ml conical tube (Corning, Falcon®, catalog number: 352070)
2. 10 ml conical tube (SARSTEDT AG & Co, catalog number: 62.9924.272)
3. 70 µm nylon cell strainer (Corning, Falcon®, catalog number: 352350)
4. 1 ml syringe (BD, catalog number: 309659)
5. 25 gauge needle (BD, catalog number: 305125)
6. 1 ml insulin syringe with 29 G needle (BD, catalog number: 326719)
7. 225 cm tissue culture flasks (Sigma-Aldrich, catalog number: CLS431082)
8. LD columns (Miltenyi Biotec, catalog number: 130-042-901)
9. LS columns (Miltenyi Biotec, catalog number: 130-042-401)
10. Vitamin K1, 10 mg/ml (International Animal Health Products, Koagulon)

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11. PBS (10x, pH 7.4, without Ca^{2+} and Mg^{2+}) (Mediatech Inc., catalog number: 46-013-CM)
12. Fetal bovine serum (FBS), heat-inactivated at 65 °C (Scientifix, catalog number: FBS-500S)
13. Bovine serum albumin (BSA) Fraction V (Amresco, catalog number: 0332-1 kg)
14. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: E5134)
15. RPMI-1640 (Thermo Fisher Scientific, Gibco™, catalog number: 11875-093)
16. Rat anti-mouse Thy1.2 clone 30H12 hybridoma supernatant (prepared in house)
17. Rat anti-mouse B220 clone RA3.6B2 hybridoma supernatant (prepared in house)
18. Rat anti-mouse CD8a clone 53-6.7 hybridoma supernatant (prepared in house)
19. Rat anti-mouse CD11b clone M1/70 hybridoma supernatant (prepared in house)
20. Rat anti-mouse erythroid lineage clone Ter119 hybridoma supernatant (prepared in house)
21. Anti-CD25 PE clone PC61 (BD Biosciences, catalog number: 553866)
22. Anti-rat IgG microbeads (Miltenyi Biotec, catalog number: 130-048-501)
23. Anti-PE microbeads (Miltenyi Biotec, catalog number: 130-048-801)
24. Recombinant mouse IL-2 (Peprotech, catalog number: 212-12)
25. Anti-IL-2 mAb clone JES6-1 (WEHI monoclonal antibody facility)
26. Sulfamethoxazole (400 mg/5 ml) and Trimethoprim (80 mg/5 ml) (Hospira, DBL, catalog number: 618670BAU)
27. MACS wash buffer (see Recipes)
28. MACS running buffer (see Recipes)
29. RPMI + 10% FBS (see Recipes)
30. IL-2/JES6-1 (see Recipes)
31. Preparation of hybridoma supernatants (see Recipes)

Equipment

1. Surgical scissors and forceps
2. 80 gauge stainless steel mesh sieves
3. MACS MultiStand (Miltenyi Biotec, catalog number: 130-042-303)
4. QuadroMACS separator (Miltenyi Biotec, catalog number: 130-090-976) or MidiMACS separator (Miltenyi Biotec, catalog number: 130-042-302)
5. 37 °C water bath
6. Benchtop centrifuge
7. Gammacell irradiator (Nordion), or equivalent
8. Heat lamp
9. Perspex mouse restrainer

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Procedure

A. Transplantation of T cell-depleted bone marrow (optional; if using immunodeficient Rag-KO hosts proceed to step B)
   1. Host mice are typically aged between 8-12 weeks of age at the time of irradiation, and are of mixed sex. Mice aged between 6-8 weeks may also be suitable hosts, although we recommend providing young mice with softened food and avoiding the use of young females with low body weight (<20 g). We use an F1 cross between C57BL/6 and B10.BR mice as host mice for allogeneic bone marrow transplantation experiments, with B10.BR bone marrow/Treg donors. Use of F1 hosts is recommended due to superior Treg reconstitution in this system. We have successfully performed syngeneic Treg reconstitution on non-F1 host mice (*e.g.*, B10.BR), but have observed inferior reconstitution using allogeneic Tregs (C57BL/6), possibly due to rejection by radio-resistant NK cells (Davis *et al.*, 2015).
   2. Inject host mice with 100 µl vitamin K (10 mg/ml solution) via subcutaneous injection in either scruff or flank 1 day prior to irradiation using an insulin syringe. We routinely give lethally irradiated mice this prophylactic treatment to prevent post-irradiation coagulopathy that has occasionally been observed in our mouse colony. Additional injections are given 3 and 7 days after irradiation.
   3. Ear mark and weigh all host mice prior to irradiation.
   4. Transfer host mice into Perspex irradiation chambers and place into an appropriate container, then transfer to irradiator.
   5. Irradiate host mice at 600Rad, then transfer back into cages.
   6. After resting mice for 3 h, perform a further irradiation of 600Rad (repeating steps A4-5).
   7. 1 day after irradiation, harvest femurs and tibias from 6-20 week old allogeneic donor mice and collect into 20 ml RPMI + 10% FBS in a 50 ml conical tube. We recommend using 1 donor mouse for every 5-8 recipients.
   8. Trim the ends off each bone and flush out bone marrow with RPMI + 10% FBS using a 1 ml syringe with a 25 gauge needle.
   9. Resuspend bone marrow cells and filter through 70 µm nylon mesh into a 50 ml conical tube.
   10. Centrifuge cells at 125 *x g* for 10 min at 4 °C, and resuspend in 1 ml MACS wash and add anti-Thy1.2 hybridoma supernatant at a concentration of 1 x 10^6 cells/1 µl supernatant. Incubate on ice for 30 min. Alternatively anti-CD3 hybridoma supernatant can be used instead of anti-Thy1.2 to bind T cells.
   11. Spin down cells at 125 *x g* for 10 min at 4 °C.
   12. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.

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13. Resuspend pellet in 1 ml MACS wash and add anti-Rat IgG microbeads at a concentration of 1 x 10^7 cells/5 µl microbead solution and incubate at 4 °C for 15 min.
14. Spin down cells at 125 *x g* for 10 min at 4 °C.
15. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
16. Resuspend cells in 1 ml MACS running buffer per 2.5 x 10^8 cells.
17. Add cells to LD column prepared according to manufacturer's instructions with a pre-separation filter. Collect effluent, and wash column with 1 ml MACS running buffer 2 times. The unbound fraction contains T cell-depleted BM cells.
18. Centrifuge T cell-depleted BM cells at 125 *x g* for 10 min at 4 °C.
19. Resuspend pellet at 16.7 x 10^6 cells/ml in RPMI for injection. Store cells on ice until ready for use.
    Note: This concentration is suitable for transfer of 5 x 10^6 cells in a volume of 300 µl. The volume used for intravenous injections may vary depending on the policies of individual institutions. If so, the concentration of cells may be adjusted to allow transfer of 5 x 10^6 cells in an alternative volume.
20. Warm host mice (irradiated the previous day) for approximately 10 min under a heat lamp to cause peripheral vasodilation, or for as long as needed to dilate the tail vein. Transfer mice to Perspex mouse restrainer. Using a 1 ml insulin syringe inject 300 µl of T cell-depleted bone marrow cells (or alternative volume containing 5 x 10^6 cells) into the tail vein of host mice. For a more detailed explanation of intravenous injections refer to Machholz *et al.* (2012).
21. The drinking water of all irradiated mice is supplemented with sulfamethoxazole (80 µg/ml) and trimethoprim (160 µg/ml) for three weeks post-irradiation.

B. Selective reconstitution of the Treg compartment of bone marrow transplant recipients or immunodeficient Rag-KO mice
    1. Wild type mice on an immunosufficient background aged between 6-20 weeks are used as donors for transfer into either allogeneic irradiated hosts (described in part A), or syngeneic immunodeficient Rag-KO hosts aged between 8-16 weeks. For transfer into Treg-reconstituted allogeneic bone marrow transplant recipients (part A) allogeneic B10.BR donors of 6-20 weeks of age are used. For transfer into Rag-KO mice several strain combinations have been tested with comparable results, including C57BL/6 donors into C57BL/6 Rag-KO hosts, B10.BR donors into B10.BR Rag-KO hosts, and [C57BL/6 x B10.BR]F1 donors into [C57BL/6 x B10.BR]F1 Rag-KO hosts. Treg reconstitution has been tested in both male and female host mice with success, but use of female host mice is avoided when Tregs are derived from male donors. A ratio of 1 donor mouse for every recipient is recommended.

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2. Remove lymph nodes and spleens from donor mice. Lymph nodes collected include the mandibular, accessory mandibular, superficial parotid, axillary, accessory axillary, subiliac, lumbar aortic, and jejunal (Van den Broeck *et al.*, 2006). Collect into 10 ml RPMI + 10% FBS in a 50 ml conical tube.
3. Prepare a single cell suspension via gentle mechanical dissociation of organs through stainless steel mesh sieves. Transfer single cell suspension to a 50 ml conical tube.
4. Centrifuge cells at 125 *x g* for 10 min at 4 °C. For enrichment of CD4 T cells, resuspend in in 1 ml MACS buffer and add anti-B220, anti-CD8α, anti-CD11b and anti-Ter119 hybridoma supernatants at a concentration of 1 x 10^6 cells/1 µl of each supernatant. Incubate on ice for 30 min.
5. Spin down cells at 125 *x g* for 10 min at 4 °C.
6. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
7. Resuspend pellet in 1 ml MACS wash and add anti-Rat IgG microbeads at a concentration of 1 x 10^7 cells/5 µl microbead solution and incubate at 4 °C for 15 min.
8. Spin down cells at 125 *x g* for 10 min at 4 °C.
9. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
10. Resuspend cells in 1 ml MACS running buffer per 2.5 x 10^8 cells.
11. Add cells to LD column prepared according to manufacturer's instructions with a pre-separation filter. Split the sample across multiple LD columns if total cell numbers exceed 5 x 10^8 cells. Collect effluent in a 10 ml conical tube, and wash column with 1 ml MACS running buffer 2 times. The unbound fraction is enriched for CD4 T cells (approximately 70% pure).
12. Centrifuge the CD4 T cell enriched fraction at 125 *x g* for 10 min at 4 °C.
13. For selection of $CD25^+$ cells from the CD4 T cell enriched fraction, resuspend cells in 1 ml MACS wash with CD25-PE at a concentration of 2 x 10^6 cells/1 µl antibody solution (*i.e.,* 0.2 µg/2 x 10^6 cells). Incubate on ice for 30 min.
14. Spin down cells at 125 *x g* for 10 min at 4 °C.
15. Discard supernatant and resuspend pellet in 10 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
16. Resuspend pellet in 1 ml MACS wash and add anti-PE microbeads at a concentration of 1 x 10^6 cells/1 µl microbead solution and incubate at 4 °C for 15 min.
17. Spin down cells at 125 *x g* for 10 min at 4 °C.
18. Discard supernatant and resuspend pellet in 10 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
19. Resuspend cells in 1 ml MACS running buffer per 2 x 10^8 cells.
20. Add cells to LS column prepared according to manufacturer's instructions with a pre-separation filter. Collect effluent in 10 ml conical tubes, and wash column with 3 ml

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MACS running buffer 3 times. Elute the CD25-enriched fraction bound to the column in 5 ml MACS running buffer into a 10 ml conical tube.

21. Spin down cells at 125 *x g* for 10 min at 4 °C.
22. Discard supernatant and resuspend pellet at 8.3 x 10^6 cells/ml in RPMI for injection into either imunnodeficient Rag-KO hosts, or lethally irradiated BMT recipients (as described in part A). If Treg cells are to be injected into irradiated hosts the purified Treg cell suspension can be mixed with bone marrow cells (step A19) to be injected simultaneously.

    Note: This concentration is suitable for transfer of 2.5 x 10^6 cells in a volume of 300 µl. The volume used for intravenous injections may vary depending on the policies of individual institutions. If so, the concentration of cells may be adjusted to allow transfer of 2.5 x 10^6 cells in an alternative volume.
23. Warm host mice under a heat lamp to cause peripheral vasodilation. Transfer mice to Perspex mouse restrainer. Using a 1 ml insulin syringe inject 300 µl of Treg cells (or alternative volume containing 2.5 x 10^6 cells) into the tail vein of host mice.
24. All host mice receive a single injection of IL-2/JES6-1 complexes (see Recipes for preparation) in a volume of 100 µl using a 1 ml insulin syringe. Administer a repeat injection 2, 4 and 6 days post-Treg transfer.

C. T cell transfer and reconstitution

1. Remove lymph nodes (mandibular, accessory mandibular, superficial parotid, axillary, accessory axillary, subiliac, lumbar aortic, and jejunal) from donor mice and collect into 10 ml RPMI + 10% FBS in a 50 ml conical tube. For transfer of 2.5 x 10^6 cells, 1 donor is sufficient to reconstitute 8-10 recipient mice. For transfer into Treg-reconstituted allogeneic bone marrow transplant recipients (parts A and B) allogeneic B10.BR donors of 6-20 weeks of age are used. For Treg-reconstituted Rag-KO mice (part B) syngeneic T cell donors of 6-20 weeks of age are used (as described for part B). Either male or female donors may be used, but transfer of male donor cells into female hosts should be avoided.
2. Prepare a single cell suspension via gentle mechanical dissociation of organs through stainless steel mesh sieves. Transfer into a 50 ml conical tube.
3. Centrifuge cells at 125 *x g* for 10 min at 4 °C.
4. For selection of CD4 T cells, resuspend pellet in 1 ml MACS buffer and add anti-B220, anti-CD8α, anti-CD11b and anti-Ter119 hybridoma supernatants at a concentration of 1 x 10^6 cells/1 µl of each supernatant. Incubate on ice for 30 min. If a mixed population of CD4 and CD8 T cells is required the anti-CD8α hybridoma supernatant can be omitted.
5. Spin down cells at 125 *x g* for 10 min at 4 °C.

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6. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
7. Resuspend pellet in 1 ml MACS wash and add anti-Rat IgG microbeads at a concentration of 5 µl/1 x 10^7 cells/5 µl microbead solution and incubate at 4 °C for 15 min.
8. Spin down cells at 125 *x g* for 10 min at 4 °C.
9. Discard supernatant and resuspend pellet in 20 ml MACS wash, then spin down cells at 125 *x g* for 10 min at 4 °C. Repeat.
10. Resuspend cells in 1 ml MACS running buffer per 2.5 x 10^8 cells.
11. Add cells to LD column prepared according to manufacturer's instructions with a pre-separation filter. Split the sample across multiple LD columns if total cell numbers exceed 5 x 10^8 cells. Collect effluent, and wash column with 1 ml MACS running buffer 2 times. The unbound fraction is enriched for T cells.
12. Spin down cells at 125 *x g* for 10 min at 4 °C.
13. Discard supernatant and resuspend pellet at 8.3 x 10^6 cells/ml in RPMI for injection. *Note: This concentration is suitable for transfer of 2.5 x 10^6 cells in a volume of 300 µl. The volume used for intravenous injections may vary depending on the policies of individual institutions. If so, the concentration of cells may be adjusted to allow transfer of 2.5 x 10^6 cells in an alternative volume.*
14. Warm host mice (from part B) under a heat lamp to cause peripheral vasodilation. Transfer mice to Perspex mouse restrainer.
15. Using a 1 ml insulin syringe inject 300 µl of purified T cell suspension (or alternative volume containing 2.5 x 10^6 cells) into the tail vein of Treg-reconstituted mice (part B) at day 7 post-Treg transfer.

Notes

1. We have found that using hybridoma supernatants in magnetic bead separation protocols to be a cost effective and flexible approach for the selection of various cell populations. However, similar results can also be achieved using commercially available antibodies. These may be either unlabeled and detected with anti-rat IgG microbeads as described in this protocol, or fluorochrome labeled and detected with mircobeads specific for the fluorochrome of interest (such as the CD25-PE positive selection described above). Alternatively, commercial kits for magnetic selection of specific cell populations such as Tregs can also be used.

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Recipes

1. MACS wash buffer
   Supplement 950 ml PBS with 10 mM EDTA and 50 ml sterile heat inactivated FBS
   Filter sterilize and stored at 4 °C
2. MACS running buffer
   Supplement 1 L PBS with 0.5% BSA and 5 mM EDTA
   Filter sterilize and stored at 4 °C
3. RPMI + 10% FBS
   Supplement 450 ml RPMI with 50 ml of sterile heat inactivated FBS
   Keep solution sterile and stored at 4 °C
4. IL-2/JES6-1
   Note: A single dose of IL-2/JES6-1 complexes consists of 1 µg IL-2/5 µg JES6-1 (approximately a 2:1 molar ratio) (Boyman et al., 2006). Stock solutions of IL-2 and JES6-1 are stored frozen at -30 °C at 1 mg/ml.
   a. To prepare 100 doses of complexes, mix 100 µg IL-2 (0.1 ml) and 500 µg JES6-1 (0.5 ml) and incubate together in a 37 °C water bath for 30 min.
   b. Aliquot into appropriate volumes for single use (6 µl per injection) and store frozen at -70 °C.
   c. Dilute to a final working concentration of 100 µl per injection in PBS immediately prior to injection.
5. Preparation of hybridoma supernatants
   a. Hybridoma cell lines are thawed and cultured in a 225 cm tissue culture flasks in RPMI + 10% FBS until dead cells begin to appear and medium is yellow (typically 7-10 days). Remove contents of flask and transfer into a 50 ml conical tube.
   b. Pellet cells by spinning at 125 *x g* for 10 min at 4 °C.
   c. Transfer supernatant into 50mL conical flasks, keeping the solution sterile. Stored at 4 °C.

   Note: We do not routinely quantify the concentration of antibody in our hybridoma supernatants. The volumes used in this protocol are in excess of what we have determined to be required using typical batches of supernatants, and are thus considered sufficient even if a particular batch of supernatant has a lower than average yield.

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