Isolation and Characterization of Murine Early Intrathymic Precursor Populations

Li Wu and Ken Shortman

1. Introduction

The earliest steps along the pathway leading to mature T cells in mouse thymus have been defined (1,2). Within the thymus, several minute but discrete populations of T cell precursors develop in sequence, preceding the stage of CD4⁺8⁺ thymocytes (Fig. 1). The earliest identifiable intrathymic precursors in the adult mouse, termed “low CD4” precursors, express low levels of CD4 and Thy-1, and are positive for Sca-1, Sca-2, CD44 and c-kit but negative for CD25 (3). This precursor population represents only 0.03–0.05% of total thymocytes. It is not exclusively T-lineage committed and retains the potential to form NK cells, B cells and dendritic cells (DC) (4–6). The low CD4 precursor population then loses surface CD4 and develops into CD3⁻4⁻8⁻ triple negative (TN) precursors. Among the TN precursors, four subpopulations, representing 2–3% of total thymocytes, can be characterized by the early expression of CD44 and c-kit, and by transient expression of CD25. The developmental progression, deduced from precursor activities, is c-kit⁺CD44⁺CD25⁻ -> c-kit⁺CD44⁺CD25⁺ -> c-kit⁻CD44⁻CD25⁺ -> c-kit⁻CD44⁻CD25⁻ (7–10). The earliest c-kit⁺CD44⁺CD25⁻ TN subpopulation, although believed to be more mature than the low CD4 precursors, has many features overlapping those of the low CD4 precursors. It also retains the potential to develop into NK cells, B cells and DC (L. Wu, unpublished observations). The next step involves the c-kit⁻CD44⁺CD25⁺ subpopulation, which has lost the potential to form NK cells and B cells, but still retains a capacity to form DC (9). It is not until the c-kit⁻CD44⁻CD25⁺ stage that the precursors are completely committed to T lineage development (9). Both c-kit⁻CD44⁺CD25⁺ and c-kit⁻CD44⁻CD25⁺ subpopulations are committed T cell precursors.
The intrathymic precursors are rare cells in adult thymus, about 97% thymocytes being either immature CD4+8+ or mature CD4+8−, CD4−8+, or CD3+CD4−8− cells. Accordingly, the principle for isolating these minute precursor populations is to maximally enrich for them prior to fluorescence activated cell sorting, in order to reduce the cost and maximize purity. This can be achieved by a large scale depletion of the mature and immature thymocytes, as well as other non-T lineage cells, using a combination of density centrifugation and immuno-magnetic bead depletion. Note that it is important to deplete non-T lineage cells, including erythrocytes, macrophages and DC, which may otherwise contaminate the precursor preparation, especially if CD44 is a sorting parameter. Although the phenotype of the low CD4 precursors and the TN c-kit+CD44+CD25+ subpopulation overlaps, the low CD4 precursor population will be partially lost if anti-CD4 antibody is included in the depletion pro-

Fig. 1. A summary of the pathway of intrathymic T cell development. The precursor sequence was deduced based on the state of TCR gene rearrangement, the precursor activity and developmental kinetics of each precursor population (3, 5, 6, 8, 9). The proportion of each precursor population amongst all thymocytes is an average value for C57BL/6 mice.
procedure, as normally used for isolating TN precursors. We therefore developed two separate procedures for purifying either the low CD4 precursors or the four TN precursor populations.

2. Materials

2.1. Mice

C57BL/6J (Ly 5.2) mice at 5–6 wk of age have been used for thymic precursor cell isolation and as donors in precursor transfer experiments. C57BL/6 Ly 5.1-Pep3b mice at age of 8–12 wk have been used as recipients in precursor transfer experiments.

2.2. Media for Single Cell Suspension and for Immuno-Fluorescent Staining

1. Balanced Salt Solution (BSS): A mouse tonicity (308m. osmolar or equivalent to 0.168 M NaCl), HEPES buffered balanced salt solution at pH 7.2 and supplemented with 3% fetal calf serum (FCS) is used for single-cell suspension and immunofluorescent staining.

2. RPMI-1640-HEPES-FCS: RPMI-1640 culture medium adjusted to mouse tonicity, buffered with HEPES at pH 6.8–7.0 and supplemented with 10% FCS is used for adhesion depletion for macrophages.

3. Fetal Calf Serum: FCS is filtered through a 0.22 micron membrane and heat-inactivated at 56°C for 30 min.

4. Density Separation Medium: Nycodenz is purchased from Nycomed Pharma AS, Oslo, Norway as analytical grade powder, 50 g bottles, MW 821. Make a stock 0.372 M (30.55 g per 100 mL final) and mix well before adjusting to final volume. Store frozen and protect from light. This stock should be close to 308m osmolar (adjust if not) and have a density about 1.16g/cm³ at 4°C. Dilute this stock with BSS, mix thoroughly, to density 1.086g/cm³ at 4°C. Use a weighing bottle to get precise density. To calculate stock dilution for the approximate density use the following formula:

\[
100 \times 1.16 \text{ (stock density)} + 1.0 \times a = (100 + a) \times 1.086 \text{ (required density)}
\]

where \( a \) = additional volume of BSS to be added to 100 mL stock. Note that the \((100 + a)\) final volume is measured after thorough mixing. Store frozen in sealed tubes or bottles. Mix thoroughly on thawing and before use. During separation maintain a temperature around 4°C to avoid density changes.

2.3. Monoclonal Antibodies for Depletion

Cocktails of monoclonal antibodies (MAb) for depletion are prepared and stored in small aliquots at −70°C (for details of each MAb, see Table 1). Each MAb is pretitrated using immunofluorescent staining with anti-Ig second stage, then is used at near saturating concentration in the final mix. The cocktail of MAb is used at 10 µL per 10⁶ cells. Two different MAb cocktails are employed.
Table 1
mAbs Used in this Procedure

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-CD3</td>
<td>KT3-1.1</td>
<td>(11)</td>
</tr>
<tr>
<td>anti-CD4</td>
<td>GK1.5</td>
<td>(12)</td>
</tr>
<tr>
<td>anti-CD8</td>
<td>53.6.7</td>
<td>(13)</td>
</tr>
<tr>
<td>anti-CD25</td>
<td>PC61</td>
<td>(14)</td>
</tr>
<tr>
<td>anti-Thy-1.2</td>
<td>30H-12</td>
<td>(15)</td>
</tr>
<tr>
<td>anti-B220</td>
<td>RA3-6B2</td>
<td>(16)</td>
</tr>
<tr>
<td>anti-Mac-1(CD11b)</td>
<td>M1/70</td>
<td>(17)</td>
</tr>
<tr>
<td>anti-Gr-1</td>
<td>RB6-8C5</td>
<td>(18)</td>
</tr>
<tr>
<td>anti-erythrocyte antigen</td>
<td>TER-119</td>
<td>From Dr T Kina, Dept Immunology, Chest Disease Research Institute, Kyoto University, Japan</td>
</tr>
<tr>
<td>anti-MHC Class II</td>
<td>M5/114</td>
<td>(19)</td>
</tr>
<tr>
<td>anti-c-kit</td>
<td>Ack-2</td>
<td>(20)</td>
</tr>
<tr>
<td>anti-NK1.1</td>
<td>DX5</td>
<td>Pharmingen, San Diego, CA</td>
</tr>
<tr>
<td>anti-Ly5.2</td>
<td>ALI-4A2</td>
<td>(21)</td>
</tr>
</tbody>
</table>

Most of these mAbs are available as purified antibody or fluorescent-conjugated antibodies from Pharmingen (San Diego, CA) or Caltag (Burlingame, CA).

1. For isolation of low CD4 precursors: anti-CD3, KT3-1.1; anti-CD8, 53.6.7; anti-CD2, RM2-1; anti-CD25, PC61; anti-B220, RA3-6B2; anti-Mac-1, M1/70; anti-Gr-1, RB6-8C5; anti-erythrocyte antigen, TER-119; anti-MHC class-II, M5/114.

2. For isolation of TN precursors: anti-CD3, KT3-1.1; anti-CD4, GK1.5; anti-CD8, 53.6.7; anti-B220, RA3-6B2; anti-Mac-1, M1/70; anti-Gr-1, RB6-8C5; anti-erythrocyte antigen, TER-119; anti-MHC class-II, M5/114.

2.4. Immunomagnetic Beads for Depletion

1. Paesel and Lorei beads: Goat anti-rat IgG coated magnetic beads are purchased from Paesel and Lorei (GMBH & Co, Frankfurt, Germany). For reasons of economy these beads are used for the first round magnetic bead depletion at a bead:cell ratio of 3:1. The beads are washed three times in 3–5 mL BSS-FCS before use, to remove the preservative which is toxic to cells. A Dynal magnet is used to recover the beads. Note that Paesel and Lorei beads are very small and therefore migrate slowly in the magnetic field. To avoid losing beads, leave the tube on the magnet for at least 3–5 min when washing the beads or recovering depleted cells.

2. Dynabeads: Sheep anti-rat Ig coated beads M450 Dynabeads (Dynal, Oslo, Norway) are used for the second round magnetic bead depletion at a bead: cell ratio of 5:1. The beads are washed three times in 3–5 mL BSS-FCS before use to remove the preservative. A Dynal magnet is used to recover the beads.
2.5. Antibodies for Immunofluorescent Staining

Fluorescent conjugated antibodies for immunofluorescent staining are either purchased from Caltag (Burlingame, CA), or Pharmingen (San Diego, CA), or are made in our laboratory. The following are used:

1. FITC-conjugated antibodies: FITC-anti-Thy-1.2 (30H-12); FITC-anti-c-kit (Ack-2); FITC-anti-Ly 5.2 (ALI-4A2).
2. PE- or Cy-3-conjugated antibodies: These can be used interchangeably in the same fluorescent channel (excitation at 488nm and emission at 570–575nm). These conjugates are: PE-anti-CD4 (GK1.5); PE-anti-B220 (RA3-6B2); Cy3-anti-CD25 (PC61).
3. APC- or Cy-5-conjugated antibodies: These can be used interchangeably in the same fluorescent channel (excitation at 605nm and emission at 660–670 nm). The conjugated antibodies are: APC-anti-c-kit (Ack-2); Cy5-anti-Mac-1 (M1/70); Cy5-anti-Gr-1 (RB6-8C5); Cy5-anti-CD8 (YTS 169.4).
4. Biotinylated antibodies: biotin-anti-Thy-1.2 (30H-12); biotin-anti-CD3 (KT3-1.1); biotin-anti-NK1.1 (DX5). Texas-Red-avidin is used as the second stage reagent.

Sorting and analysis is performed on a FACStar-Plus instrument (Becton Dickinson) or equivalent instrument permitting at least three fluorescent channel operation.

2.6. Miscellaneous Equipment

Spiral Mixer: The type with a series horizontal rollers, used for equilibrium dialysis, is set up in cold room. Fit 5 mL round-bottom Falcon tubes with a “collar” (cut from thick rubber tubing) around the lid, so the tubes will rotate slowly at an angle of ~30° when placed on the rollers.

3. Methods

3.1. Isolation of the Earliest Intrathymic Precursor Population— the “Low CD4 Precursors”

1. Single-cell suspension: A thymocyte suspension from 16 thymuses is prepared by gently forcing thymus lobes through a stainless steel sieve in BSS-3%FCS. The cell suspension is transferred into four 10 mL conical tubes (~4 thymuses per tube), underlaid with 0.5 mL FCS and centrifuged at 580 × g for 7 min at 4°C, in a benchtop centrifuge.
2. Density centrifugation: This step selects the 15–20% of thymocytes with a density less than 1.086g/cm³, including the low CD4 precursors, and removes dead cells and higher density cells, including some mature CD4⁺8⁻ and CD4⁻8⁺ thymocytes, small CD4⁺8⁺ thymocytes and erythrocytes. Transfer 5 mL of well mixed 1.086g/cm³ Nycodenz medium to four 14 mL round bottom polypropylene Falcon tubes. Resuspend the cell pellet in each conical tube in an additional 5 mL Nycodenz
medium and overlay this cell/Nycodenz suspension onto the 5 mL Nycodenz medium in the Falcon tube (i.e., cells from 4 thymuses per tube). Then layer 2 mL of FCS above the cells. Slightly mix the interface bands with a Pasteur pipet to make a gentle density gradient. Centrifuge in a swing-out rotor refrigerated centrifuge (4°C) for 10 min at 1700 g. Using a Pasteur pipet, collect the light density fraction as all upper zones down to a little below the lower interface, leaving behind the pellet and 2 mL or so of medium above it. Dilute the collected fraction with BSS to 30–40 mL, mix well, take a small sample to count cell yield at this stage, then centrifuge the cells to a pellet at 580 g for 7 min.

3. Adhesion depletion of macrophages: This step removes macrophages by adhesion to a plastic surface. Resuspend the cell pellet in 10 mL RPMI-1640-10%FCS. Transfer the cell suspension into a 10 cm plastic Petri dish and ensure even distribution over the whole area of the dish. Incubate in a 37°C CO₂-in-air incubator for 60 min. After incubation, the nonadherent cells are collected by gently washing the dish twice with 10 mL prewarmed (37°C) RPMI-1640–10% FCS (see Note 1). Take a small sample for a cell count, then collect the cells by centrifugation.

4. Immunomagnetic bead depletion: This step is to remove most cells bearing markers of mature thymocytes, of more mature precursor cells and other non-T lineage cells. Add to the cell pellet 10 µL/10⁶ cells of the depletion MAb cocktail for low CD4 precursor isolation (see Materials) and mix well. Incubate at 4°C for 30–40 min. Dilute cells in 9 mL of BSS-3%FCS, underlayer with 1 mL of FCS, then spin down cells through the FCS underlay at 580 g for 7 min. Remove the supernatant carefully from the top. Washing using a serum underlay removes residual antibody with only one washing step.

5. The antibody-coated cells are then removed using two rounds of treatment with anti-Ig coated beads. For the first round of depletion, the Paesel and Lorei beads are used for economic reasons, since large amount of beads are required at this stage. Prewash the required amount (beads : cells = 3 : 1) of anti-rat IgG coated beads in a 5 mL round bottom Falcon tube with BSS-3%FCS three times to remove the preservative. Separate the beads from the washing fluid with a Dynal magnet. Resuspend the MAb coated cells in 300–500 µL BSS-3% FCS, then transfer the suspension into a 5 mL Falcon tube containing washed beads. Mix the slurry of cells and beads, seal the tube with a cap and place a “collar” around the cap. Mix continuously for 20 min at 4°C, by rotating at a 30° angle on a spiral mixer. To recover the undepleted cells, dilute the bead-cell mix in 5 mL BSS-3%FCS, then remove beads and attached cells with Dynal magnet. Recover the bead-free cell suspension with a Pasteur pipet and once again treat with the magnet to remove any residual beads. Take a small sample of cells to count, then recover the cells by centrifugation (580 g, 7 min).

6. The second round depletion is to remove the residual antibody-coated cells, in order to obtain the maximum enrichment. Resuspend cell pellet again in 300–500 µL BSS-3%FCS, and add cells to prewashed anti-rat Ig coated Dynabeads at a ratio of bead : cell = 5 : 1. Mix the bead and cell slurry for 20 min. Dilute with
Isolation of Murine Thymic Precursor Population

2 mL BSS-3%FCS, and remove beads and attached cells with Dynal magnet. Recover the bead-free cell supernatant with a Pasteur pipet and again remove residual beads with the magnet. Recover the nondepleted cells by centrifugation at 580 g for 7 min. At this stage, the precursor cells are enriched about 500-fold, and make up ~10–20% of the cells.

7. Immunofluorescent staining and flowcytometric sorting: To obtain pure low CD4 precursors, the depleted preparation is stained in two fluorescent colors with FITC-anti-c-kit and PE-anti-Thy-1.2 (see Note 2). Propidium iodide (PI) is added to the final wash at 0.5–1.0 µg/mL. Stained cells are then analyzed on a FACStar-Plus, a file of 10,000 cells being collected. The low CD4 precursor population is represented by the Thy-1°c-kit+ subpopulation, which usually equals 10–20% of stained cells (see Fig. 2). The precursor population is sorted, setting up the live gates for Thy-1°c-kit+ cells and excluding dead cells are on the basis of very low forward scatter and positivity for PI. The purity of the sorted precursor population is determined by reanalysis on FACStar Plus and is usually >98%. The number of low CD4 precursors recovered is usually 2–3 × 10⁴ cells per thymus.

3.2. Isolation of CD3⁻4⁻8⁻ Triple Negative Precursor Populations

A procedure similar to that described above is used for isolation of the TN thymic precursor populations, except that a different depletion MAb cocktail is used. After adhesion depletion of macrophages, cells are incubated with the Fig. 2. A typical flow cytometric analysis of the depleted preparation, enriched for the low CD4 precursors. The preparation was immunofluorescent stained with FITC-anti-c-kit and PE anti-Thy-1.2. The low CD4 precursor population is represented by the Thy-1°c-kit+ cells. The box shows the sorting gates for this population.
depletion MAb cocktail for TN precursors (see Materials). This is then followed by two rounds of immunomagnetic bead depletion, as above. At this stage, the TN precursor populations are enriched 50–200-fold.

To obtain pure cells of each TN subpopulation, the depleted preparation is stained in two fluorescent colors with FITC-anti-c-kit and Cy3-anti-CD25, together with PI (see Note 3). The stained cells are analyzed on a FACStar-Plus and a file of 10,000 cells is collected. Four TN subpopulations can be segregated as seen in the contour plot (Fig. 3), namely: c-kit⁺CD25⁻ (I), c-kit⁺CD25⁺ (II), c-kit⁻CD25⁺ (III) and c-kit⁻CD25⁻ (IV), representing 3–4%, 6%, 50% and 40% of stained cells respectively. Each precursor population is sorted by setting up live gates as shown in Fig. 3. Dead cells are excluded by gating out cells with very low forward scatter and staining with PI. Purity of the sorted precursor populations is determined by reanalysis on FACStar Plus and is generally around 98–99%. The number of TN precursors recovered per initial thymus is generally: I, ~3 × 10⁴; II, ~5 × 10⁴; III, ~5 × 10⁵ and IV, ~4 × 10⁵.

3.3. Precursor Activity Analysis

1. Intrathymic transfer of isolated precursor populations: The developmental potential of each precursor population can be determined by their capability to recon-
Isolation of Murine Thymic Precursor Population

stitute T cell development in an irradiated recipient thymus lobe. Ly 5 disparate mice are used in this analysis. Eight to twelve week old C57BL/6 Ly 5.1-Pep^b recipient mice are irradiated (750 Rad, 1 Rad = 0.01 Gy) and used 1–3 h later. The irradiated mice are anesthetized by intraperitoneal injection of a mixture of Ketavet 100 (Ketamine hydrochloride 0.05mg/g body weight; Delta Veterinary Laboratories Pty.Ltd. NSW Australia) and Rompun (a muscle relaxant, Xylazine hydrochloride 0.01mg/g body weight; Bayer AG, Germany). The intrathymic injection procedure described by Goldschneider et al. (22) is used. A midline incision is made in the skin overlying the lower cervical and upper thoracic region, and the upper third of the sternum is bisected longitudinally with fine scissors to expose the thymus. A suspension (10 µL) containing the appropriate number of purified precursor cells from C57BL/6 (Ly 5.2) mice is injected directly into the anterior upper portion of each thymus lobe using a 50-µL Hamilton syringe with a 30-gauge needle (PrecisionGlide Needle 30G1, Becton Dickinson, Franklin Lakes, NJ). The incision is then closed with wound clips (MikRon Precision. Inc., NJ). The mice are kept under a warm lamp until they recover.

2. Intravenous transfer of isolated precursor populations: The potential of the precursors to develop into other hemopoietic lineages can be determined by intravenous injection into lethally irradiated Ly 5.1 recipients. Eight to twelve week old Ly 5.1 recipient mice are irradiated with two doses of 550 rads with a 3 hr interval. A suspension (200 µL) containing the appropriate number of purified precursor cells together with 5×10^4 recipient-type bone marrow cells is injected into the tail vein (see Note 4). Antibiotics are added to the drinking water (Polymyxin B sulfate 10^6u/L and Neomycin sulfate 1.1g/L) for two weeks after irradiation.

3. Analysis of progeny of transferred precursor populations: At various times after precursor transfer, the recipients are sacrificed and the thymus, spleen, lymph nodes and bone marrow are removed. Cell suspensions are prepared, and the percentage of donor and host origin cells are quantified by immunofluorescent staining and flowcytometric analysis. Cell suspensions are stained with FITC-anti-Ly 5.2 in combination with different lineage markers. For T-lineage cell reconstitution, cells are stained with FITC-anti-Ly 5.2, PE-anti-CD4, Cy5-anti-CD8 and biotin-anti-CD3 or biotin-anti-Thy-1, followed by Texas-red-avidin as second stage reagent. For reconstitution of other lineages, cells are stained with FITC-anti-Ly 5.2 together with PE-anti-B220, Cy5-anti-Mac-1 or Cy5-anti-Gr-1, and biotin-anti-NK1.1, followed by Texas-red-avidin as a second stage reagent. Donor-derived cells are revealed by gating for Ly 5.2^+ cells, then analyzing their lineage marker expression (see Note 5).

4. Notes

1. It is important to use warm but not cold medium to wash the dish, otherwise adherent cells will be released.

2. Despite the name, CD4 is generally not used in the staining and sorting to produce low CD4 precursors, since staining with a combination of Thy-1 and c-kit gives the best separation of this population.
3. CD44 expression by each precursor population is tightly correlated with the expression of c-kit, so CD44 antibody staining can be used as a sorting parameter. However, it is generally not used in order to avoid the blocking effect of anti-CD44 antibody on precursor homing in the precursor transfer analysis.

4. The recipient-type bone marrow cells are injected to ensure long-term survival of the irradiated recipients.

5. The erythroid lineage, which does not express Ly 5, cannot be monitored by this approach. Alternative approaches, such as spleen colony assay or in vitro colony formation assay, can be used.

References


Isolation of Murine Thymic Precursor Population


