# Identification and Characterization of Pro-T Lymphocytes and Lineage-Uncommitted Lymphocyte Precursors from Mice with Three Novel Surface Markers

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## Summary

The study of prethymic stages of T cell development has been limited because specific markers for mouse pro-T lymphocytes were not available. We developed a panel of rat monoclonal antibodies (mAbs) that bind to our pro-T lymphocyte clones obtained from bone marrow of young adult mice and the thymus of 14-d-old embryos. The mAbs, called Joro 30-8, Joro 37-5, and Joro 75, were found to bind to all pro-T clones tested but not to cell lines representing later stages of T cell development, B lymphocyte, or myeloid lineages. We determined the frequency and tissue distribution in normal and immunodeficient mouse strains as well as the ontogeny in liver and thymus of cells positive for these mAbs. The results were consistent with the pattern of reactivity observed with cell lines. We isolated Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ bone marrow cells by cell sorter and found that: (*a*) phenotypically, they are Thy-1$^+$, CD4$^-$, CD8$^-$, CD3$^-$, B-220$^-$, IgM$^-$, F4/80$^-$, and PgP-1$^+$; (*b*) they grew in response to the combination of interleukin 3 (IL-3) + IL-4 or IL-3 + IL-4 + IL-6; and (*c*) Joro 37-5$^+$ and Joro 75$^+$ marrow cells gave rise to mature T lymphocytes but not to B lymphocytes, while Joro 30-8$^+$ marrow cells generated both T and B lymphocytes after 8–12 wk of transfer into severe combined immunodeficient (Scid) mice. In normal mice subjected to 600 rad of irradiation to induce a wave of thymus recolonization, we found by flow fluorocytometry analysis that Joro$^+$ cells entered the thymus 2 d after irradiation, expanded during the next 4 d, and underwent further differentiation, and from day 8 up to day 21, post-irradiation Joro$^+$ cells were no longer detectable in the thymuses. Immunohistochemical analysis of normal thymus shows the presence of very few Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ lymphoid cells in the subcapsular area and outer cortex but not in the medulla. The kinetic analysis of tissue sections from thymuses at various days post-irradiation suggests that Joro$^+$ cells enter the thymus via blood vessels through the subcapsular and outer cortex areas; subsequently, these cells seem to migrate to the inner cortex without reaching the medulla, and give rise to Joro$^-$ thymocytes. We conclude that in bone marrow of young mice, cells expressing Joro 30-8 are very early hematopoietic precursor cells that are either committed to the lymphocyte pathway or are even less differentiated pluripotent cells. Commitment to T cell differentiation appears to be associated with additional expression of Joro 37-5 and Joro 75.

During mouse development, hematopoietic precursor cells from fetal liver colonize the thymus; in postnatal life, the thymus is seeded by precursor cells from the bone marrow (1–4). The immigrant precursors proliferate and differentiate in this environment, giving rise to thymocytes and eventually to functional peripheral T lymphocytes. The process of T cell differentiation within the thymus has been studied by cell surface phenotyping and the analysis of TCR gene rearrangements (5–10). However, the study of prethymic stages of T cell development has been limited because specific markers for mouse T cell progenitors were not available. Recently, it has been found that a small population of bone marrow cells (0.1–0.3%) expressing Thy-1 and Sca-1 surface antigens, and lacking surface markers expressed by T and B lymphocytes or myeloid cells, possessed thymus-repopulating potential (11). However, neither Thy-1 nor Sca-1 are specific markers for T cell progenitors and/or pluripotent stem cells (12, and our unpublished results).

Over the last 5 yr, we have established several T cell progenitor clones from the bone marrow of young adult mice and the thymus of 14-d-old mouse embryos. These clones represent the two earliest stages of T cell development identified so far. We call them "marrow-type" and "thymus-type" pro-T lymphocytes (reviewed in reference 13). Both types of clones contain the TCR $\alpha$, $\beta$, $\gamma$, and $\delta$ genes in the germline configuration. Thymus-type clones express mRNA from

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the CD3γ, but not from the CD3δ or CD3ε genes. Both types of pro-T clones are Thy-1$^+$ Sca-1$^-$. Marrow-type clones grow in vitro in the presence of IL-3 and IL-4, and thymus-type clones grow in IL-2 or IL-4 (14–17). Both types of clones generate in vitro all major subsets of thymocytes, including cells expressing TCR-α/β–CD3, and cells expressing TCR-γ/δ–CD3 receptor complexes on the cell membrane, if they are cultured in the presence of thymic epithelial cells (18).

We have developed a panel of rat mAbs against these clones in order to be able to identify these cells in vivo, to isolate them, and to study their functional properties. Three mAbs (Joro 30-8, Joro 37-5, and Joro 75) that were specific for unique cell surface determinants of early T cell progenitors were studied in detail and are the subject of the present report.

## Materials and Methods

*Animals.* BALB/c, C57BL/6, and CBA/J normal female mice (3–12 wk old) were from IFFA Credo (Saint-Germain, France). BALB/c and C57BL/6 *nu/nu* mice (6–8 wk old) were from Bomholgard (Ry, Denmark). (NIH Swiss II *nu/nu* × CBA/N)$F_1$ (*xid/nu*) female (6–8 wk old) mice, C.B. 17 Scid, and AKR Scid (6–12 wk old) mice are bred and housed in sterile isolators in the animal barrier facility of our institute. Scid mice without detectable serum Ig were used. C57BL/6 pregnant mice were from our breeding facility. The day of detection of vaginal plug was considered day 0 of gestation.

*Cell Preparation.* Cell suspensions were prepared free of erythrocytes from thymus, spleen, lymph nodes, bone marrow, and fetal liver, as described (14, 19, 20). Cells were washed and resuspended in appropriate buffer or culture medium (IMDM + 5% FCS + 2-ME [$5 \times 10^{-5}$ M], L-glutamine [2 mM], gentamycin [50 μg/ml]) at the desired concentrations.

*Cell Lines.* Growth factor–dependent lines included bone marrow pro-T lymphocyte clones (C4-77, C4-86, C4-95) (14); fetal thymocyte clones (FTH5, FTF1, FTA2, FTG12, FTH12, FTD11) (16, 17); bone marrow pro-B lymphocyte clones (CB/Bm7, BC/Bm11, LyD9, LyH7) (19, 20); CD8$^-$ CD4$^-$ TCR-α/β–CD3$^+$ LD1 T cell line (21); myeloid progenitor bone marrow clone Mye-5 (established with IL-3 from bone marrow of CBA/j mice) (Palacios, R., unpublished results); IL-3-dependent mast cell line (32Dcl) (22); IL-2-dependent cytolytic T cell lines (CTLL, CFL1) (23, 24); and Th cell line HT-2 (25). Tumor cell lines included thymic lymphomas BW5147, EL-4; fetal liver pre-B cell lymphoma CgC (provided by S. Carson, Basel Institute for Immunology, Basel); marrow pre-B cell lymphomas (18.81, 40E1, 70Z/3); B cell lymphomas (BCL1, WEHI-279, WEHI231); macrophage cell line (P388D1); mastocytoma (P815), myelomonocytic leukemia (WEHI-3), fibroblast lines (L, 3T3).

*Development of Joro Hybridomas.* Two Lewis rats were injected each with $2 \times 10^7$ C4-77 pro-T cells (emulsified in CFA) in the right hind foot pad. 3 d later, $2 \times 10^7$ C4-77 cells were injected (without adjuvant) in the same foot pad. 4 d after the second injection, $2 \times 10^7$ C4-77 cells (without adjuvant) were injected again in the same region. On the next day, the regional lymph nodes from one of the rats were obtained, cell suspensions were prepared, and fused with the Ag8.653 myeloma cells as described (26). Supernatants from wells clearly containing growing hybridoma cells were tested by flow fluorocytometry (FM)[1] analysis for reactivity with the C4-77 pro-T lymphocyte clone. Supernatants that scored positive on these cells were subsequently screened on a pro-B lymphocyte clone (CB/Bm7), a pre-B cell lymphoma (18.81), a macrophage line (P388D1), total thymocytes from adult mice, and 15-d fetal liver mononuclear cells from CB57BL/6 embryos. Aliquots of all these Joro hybridomas were frozen. Three hybridomas called Joro 30-8, Joro 37-5, and Joro 75 were selected for further study. They were recloned twice by limiting dilution (0.5 cells/well) in culture medium supplemented with IL-6. The class of rat Ig produced by the hybridomas was determined by ELISA with a commercially available kit (Amersham Corp., Arlington Heights, IL). Joro 30-8–, Joro 37-5–, and Joro 75–purified mAbs were conjugated to biotin-*N*-hydroxysuccinimide, as described before (27).

*Other Antibodies.* Biotin- or FITC-conjugated antibodies were used against: B-220 (hybridoma 14.8), IgM$^{a,j}$ (hybridoma RS3.1), CD3 (hybridoma 145-C11), Thy-1.2 (hybridoma 30H12), CD8 (hybridoma 5367.2), CD4 (hybridoma GK1.5). FITC-LyT2 and PE-L3T4 were purchased from Becton Dickinson & Co. (Mountain View, CA). The following antibodies were used in the form of hybridoma culture supernatant: j11d, B-220 (hybridoma RA3-3A1), M5/114 (IA, IE), PgP-1, (I42/5), GM1.2 (granulocytes), and F4/80 (macrophages). The reports describing the development and characterization of these mAbs are listed elsewhere (14, 19, 20, 27). Second-step reagents in FM analysis (see below) were: FITC-labeled species-specific anti-rat IgG1, IgG2a, IgG2b, and IgM from the Binding Site Ltd. (Birmingham, England). FITC-conjugated anti-mouse μ, anti-rat Ig were from Southern Biotechnology Associates (Birmingham, AL). Biotin-labeled anti-rabbit IgG and FITC-labeled streptavidin were from Amersham Corp.

*Cytokines.* rIL-1β (kindly provided by M. Brockhaus, Hoffmann-La Roche & Co. Ltd., Basel). Supernatants from X63Ag8 myeloma cells transfected with cDNAs coding for IL-2, IL-3, IL-4, IL-5, and IL-6 (28) and tested for biologic activity in proliferative assays, as described (20, 29), were used. 1 U of activity was considered to the dilution of the supernatant given half of the maximal responses. rIL-7 (30) was a kind gift of Dr. S. Gillis (Immunex Corp., Seattle, WA).

*Purification of Joro$^+$ Bone Marrow Cells.* Bone marrow cells from 3-wk-old CBA/J mice were depleted of B-220$^+$, j11d$^+$, Ia$^+$, and GM1.2$^+$ cells by treatment with a mixture of cytotoxic mAbs (RA3-3A1, j11d, M5/114, GM1.2) and complement as described (14, 20, 27). This treatment killed 91–98% nucleated bone marrow cells, as assessed by trypan blue dye test. Purification of Joro 30-8$^+$, Joro 37-5$^+$, or Joro 75$^+$ marrow cells was carried out by cell sorter (FACS 440; Becton Dickinson & Co.) using biotin-labeled Joro mAbs and FITC-streptavidin (27). After completion of the cell sort, a fraction of the sorted cells were reanalyzed to assess the degree of purification obtained. The results reported here are from experiments performed with marrow cell preparations in which the highest degree of purification (99.0–99.5%) could be obtained (see also Results and Discussion).

*Assay for Cell Proliferation.* Cell sorter–purified Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ marrow cells were cultured at $5 \times 10^3$ cells/well in round-bottomed microplates (Nunc, Roskilde, Denmark) containing saturating concentrations of the various ILs (100 U/ml) (from rIL-1β to rIL-7) either alone or in several combinations in a final volume of 200 μl of culture medium. Cultures in triplicate were incubated at 37°C, and cell growth was assessed both visually (with an inverted microscope) and by [$^3$H]thymidine uptake (1 μCi/well, sp act 185 MBq) (The Radiochemical Centre, Amersham Corp.) during the last 8 h of a 3-d-culture period performed at 37°C. The results are presented as cpm × $10^{-3}$ and represent the mean of triplicate wells per group.

[1] *Abbreviation used in this paper*: FM, flow fluorocytometry.

*Assays for Functional Potential of Joro$^+$ Cells.* AKR scid mice exposed to 350 rad (140 rad/min) of $\gamma$ rays (gamma cell 40; Atomic Energy of Canada Ltd., Ontario) 20–24 h before were injected intravenously with cell sorter-purified Joro$^+$ marrow cells (2–2.5 × $10^5$ cells/0.4 ml PBS/mouse) or PBS alone (controls). Before injection into mice, the cell sorter-purified Joro$^+$ marrow cells were incubated in culture medium supplemented with rIL-3 + rIL-4 at 37°C for 12 h to allow shedding of bound antibodies. Mice were kept in cages with individual sterile filters in sterile isolators and were fed with sterile food and water containing antibiotics.

In the experiments carried out to assess thymus recolonization potential of Joro$^+$ cells, C57BL/6 mice were exposed to 600 rad of $\gamma$ rays (140 rad/min) to induce a wave of thymus recolonization. The presence of Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells in either thymocyte cell suspensions was assessed by FM analysis daily during 21 d using biotin-labeled Joro antibodies and FITC-streptavidin, or in thymic tissue, sections were determined by immunoperoxidase staining (see below). Three mice were studied at each time point.

*FM Analysis.* Immunofluorescence staining and FM analysis was performed with a FACScan or a FACS I analyzer instrument (Becton Dickinson & Co.) as described (18, 19, 27). Negative controls were cells incubated with biotin-labeled anti-rabbit IgG followed by FITC-streptavidin, and cells incubated with second-step reagents only. Dead cells were excluded from analysis with propidium iodide. Fluorescence emitted by single viable cells was measured using logarithmic amplification. Two-color FM analysis was carried out as detailed elsewhere (18), viable cells were identified by using a combination of forward and side scatters. Thymocytes from normal BALB/c or C57BL/6 mice were used as a positive control and to set up electronical compensations for red and green fluorescence. Data from 1–5 × $10^4$ cells were analyzed with Consort 30 software.

*Immunoperoxidase Analysis.* Frozen sections were cut from thymuses of both normal C57BL/6 mice and mice exposed to sublethal irradiation, placed on glass slides, fixed in acetone, and dried at −30°C. Immunoperoxidase staining was carried out as described (31) using biotinylated goat anti-rat Ig, the avidin-biotin peroxidase complex, and 3.3'-diaminobenzidine (31). After this, the slides were stained with Giemsa and processed for microscopic examination (31).

## Results and Discussion

*Development of Joro Hybridomas.* A Lewis rat was immunized with C4-77 marrow pro-T cells in CFA in the footpad and boosted 3 and 7 d later with these cells without adjuvant. 1 d after the last injection, the regional cells were obtained and fused with the HAT-resistant Ag8.653 myeloma. Out of 956 hybridomas tested, 184 produced antibodies that bound to the marrow pro-T clone C4-77. The latter mAbs were further screened by immunofluorescence staining and FM for their reactivity with a marrow pro-B lymphocyte clone (CB/Bm7), a pre-B cell lymphoma (18.81), a macrophage tumor line (P388D1), total thymocytes from adult mice, and mononuclear cells from 15-d-old fetal liver. Several potentially interesting mAbs were found. In Table 1, they are grouped according to their pattern of reactivities on the various cells tested. This report describes detailed studies with three mAbs from group 1 called Joro 30-8 (IgM), Joro 37-5 (IgG2a), and Joro 75 (IgG2a). A preliminary account of this work has been published in a recent review (13).

**Table 1.** *Screening of Joro Hybridomas*

| | Pattern of reactivity | | | | | | |
|---|---|---|---|---|---|---|---|
| Group | Pro-T | Pro-B | Pre-B | MØ | A.T. | F.L. | No. of hybridomas |
| 1 | + | − | − | − | ± | ± | 10 |
| 2 | + | − | − | + | − | ± | 3 |
| 3 | + | + | − | − | − | ± | 105 |
| 4 | + | + | − | − | + | ± | 42 |
| 5 | + | + | + | − | ± | ± | 2 |
| 6 | + | + | − | + | ± | ± | 17 |
| 7 | + | + | + | + | ± | ± | 5 |
| 8 | − | | | | | | 772 |

The grouping of the Joro hybridomas was made according to their reactivity (assessed by FM) with Pro-T (C4.77), Pro-B (CB/Bm7), Pre-B (18.81), MØ (P388D1) lines, total thymocytes from adult mice (A.T.) and mononuclear cells from 15-d fetal liver (F.L.). The groups can further be subdivided according to the presence or absence of positive cells detected in adult thymus and fetal liver (not shown).

*Reactivity of Joro mAbs with Cell Lines.* The specificity of the Joro 30-8, Joro 37-5, and Joro 75 mAbs was determined by FM using a panel of cell lines representing different hematopoietic lineages and distinct stages of T or B lymphocyte development. The Joro 177 mAb that binds to all cells tested was included in these studies as a positive control. The Joro 30-8, Joro 37-5, and Joro 75 mAbs bound to all marrow pro-T clones (C4-77, C4-86, C4-95) and to all thymus-type pro-T clones (FTH5, FTF1, FTG12, FTA2, FTH12, FTD11) tested. However, they did not stain cell lines representing later stages of T cell development nor any other hematopoietic cell types. The following cell lines were found to be negative: thymic lymphomas BW5147 and EL-4, a CD4$^-$CD8$^-$ TCR-$\alpha/\beta$-CD3$^+$ T cell line (LD1), cytolytic T cell lines (CTLL, CFL1), a Th cell line (HT-2), pro-B lymphocyte clones (LyD9, Bc/Bm11, LyH7, CB/Bm7), a fetal liver pre-B cell lymphoma (CgC), marrow pre-B lymphomas (18.81, 40E1, 70Z/3), mature B lymphomas (BCL1, WEHI279, WEHI231), plasmacytomas (HOPCI, SP2/0, X63Ag8), a macrophage cell line (P388D1), a myelomonocytic cell line (WEHI-3), a mastocytoma (P815), IL-3-dependent myeloid lines (32Dcl, Mye5), and fibroblast cell lines (L, 3T3). Representative examples are shown in Fig. 1. These analyses show that the Joro 30-8, Joro 37-5, and Joro 75 mAbs bind to pro-T lymphocyte clones but not to cell lines representing either later stages of T cell development or other hematopoietic lineages.

*Identification of Joro$^+$ Cells in Tissues of Adult Mice and Embryos.* Cell suspensions from various tissues of adult (6–12 wk old) normal mice as well as of immunodeficient mice carrying the nude, Scid, or both xid and nude (*xid/nu*) muta-

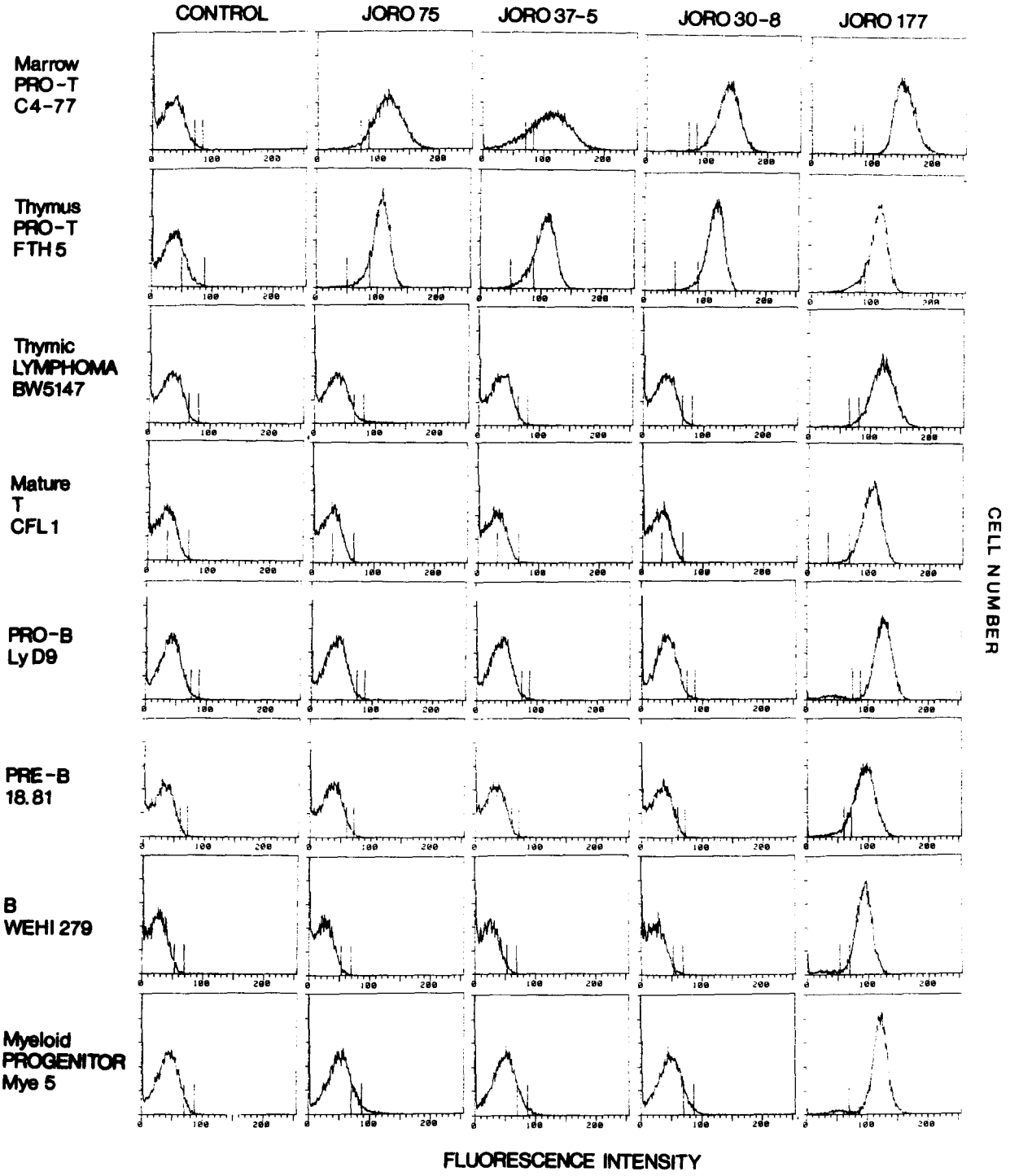


**Figure 1.** Binding of the Joro mAbs to cell lines representing different hematopoietic lineages and distinct stages of T and B lymphocyte development was studied by FM analysis using biotin-labeled Joro mAbs followed by FITC-streptavidin. The Joro 177 mAb served as positive control.

tions was studied by FM analysis. Joro$^+$ cells (<1%) were not detected in lymph nodes, thymus, bone marrow, nor in spleen of normal adult mice (BALB/c, C57BL/6, CBA/J). Nude mice had in the bone marrow 1–4% cells, and in the spleen, 1–3% cells that bound the three Joro mAbs, and *xid/Nu* mice had 2–5% Joro cells in the marrow and 1–3% in the spleen. CD17 Scid mice had no detectable (<1%) positive cells in the marrow and 2–4% spleen cells reactive with the three Joro mAbs.

Next, we studied the liver and thymus from C57BL/6 embryos. Table 2 summarizes the results of these experiments. The three Joro mAbs bound to 1–3% nucleated fetal liver

**Table 2.** *Ontogeny in Liver and Thymus from C57BL/6 Mice of Cells Recognized by the Joro 30-8, Joro 37-5, and Joro 75 mAbs*

| Organ | Day of gestation | Percent positive cells (FM analysis) | | |
|---|---|---|---|---|
| | | Joro 30-8 | Joro 37-5 | Joro 75 |
| Liver | 14 | <2* | <2* | 2–3* |
| | 15 | 2–5 | 3–5 | 4–5 |
| | 16 | <2 | 2–3 | 2–3 |
| | 17 | <2 | <2 | <2 |
| | 18 | <1 | <1 | <1 |
| Thymus | 14 | <2 | 73–85 | <2 |
| | 15 | <2 | 67–82 | <2 |
| | 16 | <2 | 25–39 | <2 |
| | 17 | <1 | 14–18 | <1 |
| | 18 | <1 | <2 | <1 |
| | Young adult | <1 | <1 | <1 |

* Range of positive cells detected in three separate experiments. FM analysis was carried out using biotin-conjugated Joro antibodies and FITC-strepavidin.

cells from 14-d mouse embryos, with ≤5% at day 15 and with ≤2% up to day 18 of gestation.

Joro 30-8 and Joro 75 mAbs bound to ≤2%, and Joro 37-5 mAb bound to 73–85% thymocytes from day 14 mouse embryos. Joro 37-5$^+$ thymocytes decreased to <40% at day 16, 14–18% at day 17, and to <1% in thymuses from young mice. Joro 30-8$^+$ and Joro 75$^+$ fetal thymocytes remained at ≤2% from day 16 until birth.

The frequency and tissue distribution of cells that bind Joro 30-8, Joro 37-5, and Joro 75 mAbs are consistent with the results obtained with cell lines and strengthen the view that these antibodies recognize early T cell precursors, but not most of the adult thymocytes, mature T cells, B cell precursors, mature B lymphocytes, or cells of the myeloid lineage. In addition, these results point out that athymic nude mice have increased numbers of T cell precursors in the bone marrow and spleen as compared with normal mice.

*Purification of Joro$^+$ Bone Marrow Cells.* To be able to study the phenotype and functional properties of Joro$^+$ cells, we isolated these cells from the bone marrow of 3-wk-old CBA/J mice. First, we depleted bone marrow cell suspensions of irrelevant cells by treatment with a mixture of cytotoxic mAbs and complement, and then we isolated Joro$^+$ cells using the FACS (see Materials and Methods for details). Fig. 2 shows with some examples that after antibody plus complement killing of irrelevant cells (91–98% nucleated cells), Joro$^+$ cells (range 15–37%) were clearly detectable, whereas no Joro$^+$ cells (<1%) were found in marrow cell suspensions before such treatment. After cell sorting, highly enriched populations of Joro$^+$ cells (range 87.8–99.5%) could be obtained. The experiments described below were performed with Joro$^+$ marrow cell preparations in which the highest degree of purification (99–99.5%) could be obtained (Fig. 2).

*Phenotype of Joro$^+$ Marrow Cells.* After an incubation period at 37°C for 8–12 h, the cell sorter–purified Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells were washed and phenotyped by FM analysis using a panel of mAbs specific for cells of the T, B, or myeloid lineages. The cell sorter–purified Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ marrow cells express low levels of Thy-1, but not CD8, CD4, and CD3 T cell lineage markers. They are positive for PgP-1 and negative for IgM, B-220, Ia, and F4/80 surface markers normally expressed by cells of the B lymphocyte and the myeloid lineages. Interestingly, ~7–15% of the Joro 30-8$^+$–purified marrow cells do not bind the Joro 75 and Joro 37-5 mAbs, whereas all Joro 75$^+$– and Joro 37-5$^+$–purified marrow cells bind the Joro 30-8 mAb. Thus, there are at least two subsets of Joro 30-8$^+$ marrow cells, a small subset that lacks and a larger one that expresses the molecules recognized by the Joro 37-5 and Joro 75 mAbs.

*Growth Requirements of Joro$^+$ Marrow Cells.* The proliferative responses of cell sorter–purified Joro$^+$ marrow cells from CBA/J mice to saturating concentrations of rILs alone or in several combinations were assessed by [$^3$H]thymidine uptake during the last 8 h of a 3-d culture period. rIL-3 and rIL-4 each promoted some proliferation of Joro 30-8$^+$, Joro 37-5$^+$, or Joro 75$^+$ marrow cells, while rIL-1, rIL-2, rIL-5, rIL-6, and rIL-7 did not. The combinations of rIL-3 + rIL-4 and rIL-3 + rIL-4 + rIL-6 gave the best responses (Fig. 3). Confirming these results, we have been able to establish continuously proliferating clones from Joro 30-8$^+$ and Joro 75$^+$ marrow cells with rIL-3 + rIL-4 and rIL-3 + rIL-4 + rIL-6 (Palacios, R., and J. Samaridis, manuscript in preparation).

*Functional Potential of Joro$^+$ Marrow Cells In Vivo.* The potential of cell sorter–purified Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ bone marrow cells to develop into mature T and/or B lymphocytes from CBA/J mice was studied by injecting them into sublethally irradiated (350 rad) T and B cell–deficient AKR Scid mice. In this experimental system, the presence of Thy-1.2$^+$ and/or IgM$^{b+}$ lymphocytes detected by allotype-specific mAbs in the recipient AKR Scid mice (Thy-1.1, IgM$^d$ allotypes) mark T and B lymphocytes, respectively, which originate from the donor Joro$^+$ marrow cells from CBA/J mice (Thy-1.2, IgM$^b$ allotypes). Thymocyte cell suspensions from Scid mice that had received Joro 30-8$^+$–, Joro 75$^+$–, or Joro 37-5$^+$–purified marrow cells contained CD4$^+$8$^-$, CD4$^+$8$^+$, and CD4$^-$8$^+$ thymocytes, a proportion of which were also CD3$^+$ (Fig. 4, *B–D*). Thymus from Scid mice that received no marrow cells did not contain such thymocyte populations (Fig. 4 *A*). Mononuclear spleen cells from AKR Scid mice that received cell sorter–purified Joro$^+$ marrow cells or no cells (control) were obtained 8–12 wk after transfer of the Joro$^+$ marrow cells. The presence of T lymphocytes (Thy-1.2$^+$, CD8$^+$, CD4$^+$, CD3$^+$) and B lymphocyte lineage cells (B-220$^+$, IgM$^{b+}$) was determined by FM analysis. Table 3 summarizes the results obtained in the four mice per group studied. Spleen cells from AKR Scid mice injected with Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ marrow cells contained Thy-1.2$^+$, CD4$^+$, CD3$^+$ and Thy-1.2$^+$,

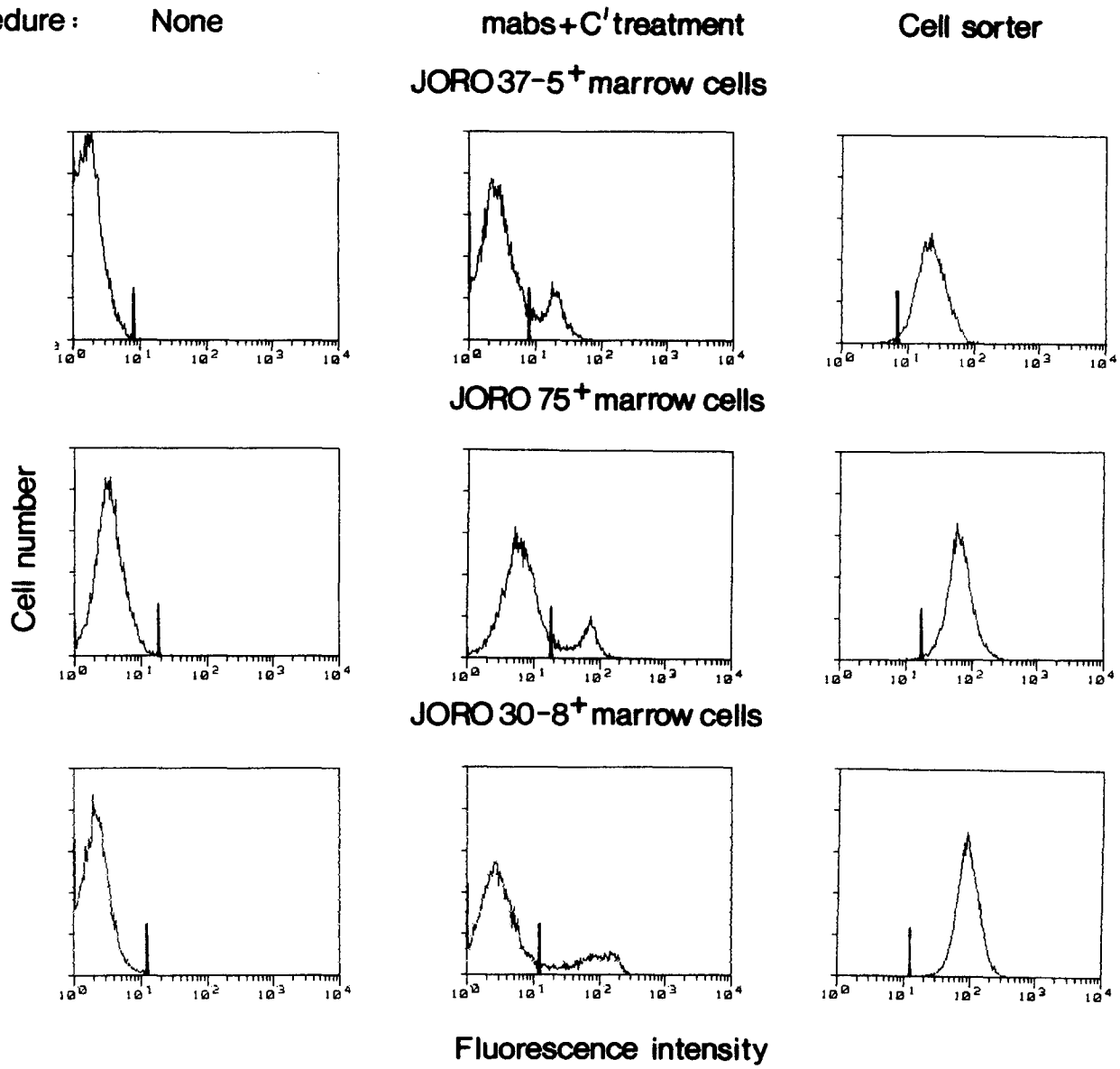


**Figure 2.** The percentage of Joro 30-8$^+$, Joro 37-5$^+$, or Joro 75$^+$ cells in marrow cell preparations treated or left untreated with a mixture of mAbs + C to eliminate irrelevant cells or after completion of the cell sort procedure was determined by FM analysis. Untreated and mAbs + C'-treated marrow cells were stained with biotin-labeled Joro antibody followed by FITC-streptavidin. Cell sorter positively selected Joro 30-8$^+$, Joro 37-5$^+$, or Joro 75$^+$ cells were reanalyzed after completion of the sort procedure. The data shown above were from experiments in which the best degree of purification was obtained. These cell sorter-purified Joro$^+$ marrow cell preparations were used in the experiments summarized in Table 3 and Fig. 3.

CD8$^+$, CD3$^+$ T lymphocytes. Spleen cells from mice that received Joro 30-8$^+$ marrow cells, but not from those mice that received Joro 37-5$^+$ or Joro 75$^+$ marrow cells, also have B-220$^+$, IgM$^{i+}$ B lymphocytes. Control AKR Scid mice that received no cells had no detectable Thy-1.2$^+$, CD8$^+$, CD4$^+$, CD3$^+$ T cells nor IgM$^{i+}$, B-220$^+$ (<1–4.5%) B lymphocytes. Thus, Joro 37-5$^+$ and Joro 75$^+$ marrow cells seem to be functionally restricted to develop along the T lymphocyte pathway, i.e., they are pro-T lymphocytes. Further in vitro experiments are in progress with Joro 75$^+$ and Joro 37-5$^+$ marrow cells to subject the apparent restricted potential of these cells to a more stringent test.

The Joro 30-8$^+$ marrow cell population seems to comprise both pro-T lymphocytes (all Joro 75$^+$– and Joro 37-5$^+$–purified marrow cells are Joro 30-8$^+$) and lineage-uncommitted lymphocyte precursors. The latter conclusion is based on the following grounds: (*a*) Marrow cells representing the earliest stage of B cell development (before Ig gene rearrangement), i.e., pro-B lymphocytes, are Joro 30-8 negative (Fig. 1). (*b*) The Joro 30-8$^+$ marrow cell population comprises at least

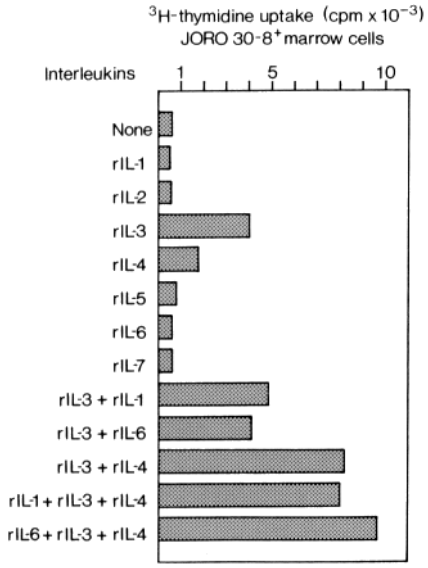


**Figure 3.** Proliferative response to recombinant ILs of cell sorter-purified Joro 30-8$^+$, Joro 37-5$^+$, or Joro 75$^+$ marrow cells. The results obtained with purified Joro 30-8$^+$ marrow cells are only shown above, as similar results were obtained with purified Joro 75$^+$ and Joro 37-5$^+$ marrow cells.

two subpopulations: Joro 30-8$^+$, 75$^-$, 37-5$^-$, and Joro 30-8$^+$; and 37-5$^+$ and 75$^+$. (*c*) We have established in culture Joro 30-8$^+$, 37-5$^-$, and 75$^-$ bone marrow clones from 3-wk-old CBA/J mice and find that they can generate both T and B lymphocytes in vitro and in vivo (Palacios, R., and J. Samaridis, manuscript in preparation).

Thus, Joro 30-8$^+$, 37-5$^+$, 75$^+$ marrow subset can give rise to T lymphocytes, while Joro 30-8$^+$, 37-5$^-$, 75$^-$ subset can generate both T and B lymphocytes. We do not know yet whether this latter subset can give rise also to myeloid cells. Experiments with the Joro 30-8$^+$, 37-5$^-$, and 75$^-$ bone marrow clones recently established in our laboratory are in progress to directly address this issue.

*Thymus Colonization by Joro$^+$ Marrow Cells.* To study thymus colonization by Joro$^+$ cells, we chose the following experimental system. Normal young adult C57BL/6 mice were exposed to a sublethal dose of irradiation (600 rad) to induce a wave of thymus recolonization. As Joro$^+$ cells are not detectable by FM analysis in thymus of young adult mice, it was possible to follow the entry of T cell progenitors into the thymus by assessing the presence of Joro$^+$ cells in the thymus at different times post-irradiation. This was carried out daily by FM analysis during a period of 21 d and by studying three mice at each time point. Fig. 5 summarizes the results of this experiment. Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells were detected in the thymuses from day 2 post-irradiation. The frequency of cells positive for all three Joro antibodies remained relatively constant up to day 6, decreased by day 7, and Joro$^+$ cells could not be detected on any of the following 13 d. The absolute number of Joro$^+$ thymocytes increased from $1.08 \times 10^4$, $1.32 \times 10^4$, and $1.44 \times 10^4$ (Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells, respectively) at day 2, to $1.25 \times 10^5$, $1.72 \times 10^5$, and $1.67 \times 10^5$ Joro$^+$ cells at day 6 post-irradiation. There was a good correlation among the Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells entering the thymus at any time point during the 5-d period of colonization (Fig. 5), which is compatible with the view that the colonizing cells bear all three Joro surface markers. CD4$^+$8$^-$ and CD4$^+$8$^+$ thymocytes were observed from day 5–6, and CD4$^-$8$^+$ thymocytes were detected from day 9–10 post-irradiation (Fig. 5 *B*, and data not shown). These findings show that Joro$^+$ cells enter the thymus 2 d after irradiation, where they expand during the following 4 d and undergo further differentiation.

In another experiment following the same protocol as above, the presence and the localization of Joro$^+$ cells was determined by immunoperoxidase staining of tissue sections from both normal thymus and thymuses obtained daily during the first 8 d after sublethal irradiation of C57BL/6 mice. Very few Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells were found in the thymus of normal C57BL/6 mice. Virtually all Joro$^+$ cells were in the subcapsular area and in the outer cortex,

**Table 3.** *Functional Potential of Cell Sorter-purified Joro$^+$ Marrow Cells In Vivo*

| Donor marrow cells | Percent positive in total spleen cells (FM analysis) | | | | | |
|---|---|---|---|---|---|---|
| | Thy-1.2 | CD8 | CD4 | CD3 | IgM | B-220 |
| None | <1 | <1–2 | <1 | <1 | <1 | <1–4.5 |
| Joro 30-8$^+$ | 10.4–18.8 | 4–7.5 | 5.3–8.7 | 9.1–13.9 | 5–13.7 | 5.6–14.3 |
| Joro 37-5$^+$ | 14–19.7 | 4.5–8.9 | 4.7–12.1 | 12.2–16.8 | <1 | <1–3 |
| Joro 75$^+$ | 9.1–20.5 | 5.6–9.4 | 3.2–9.3 | 9.0–18.0 | <1 | <1–4 |

Cell sorter-purified Joro$^+$ marrow cells from 3-wk-old CBA/J mice (see legend to Fig. 2) or PBS alone (control) were injected intravenously into sublethally irradiated AKR Scid mice. 8–12 wk later, spleen cells from the different groups of mice were tested FM analysis for the presence of cells expressing the surface markers indicated above. The numbers are the range of positive cells observed in the four mice per group studied.

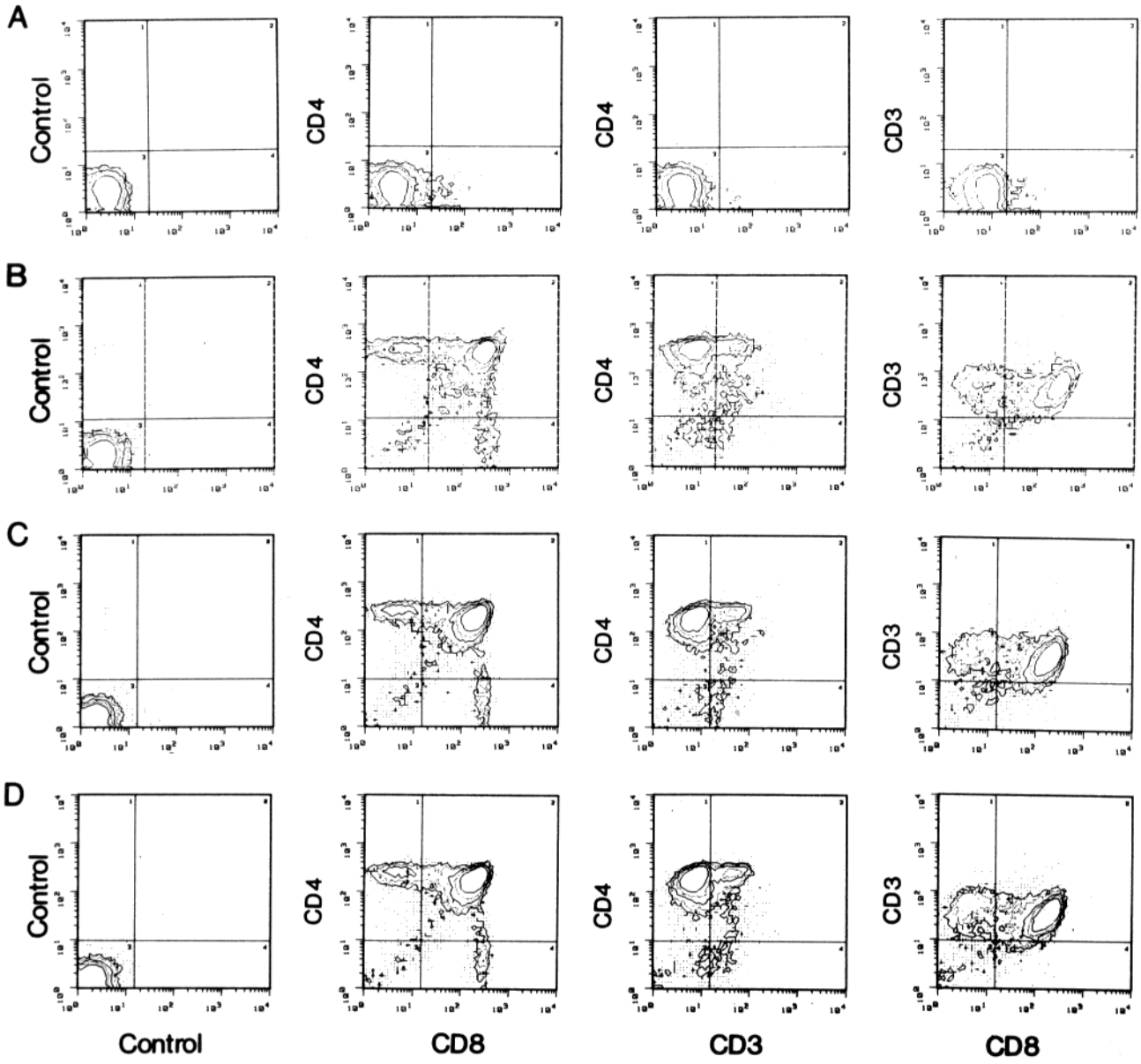


**Figure 4.** Two-color FM analysis of thymus from scid mice that had received Joro 30-8$^+$ marrow cells (*B*), Joro 37-5$^+$ marrow cells (*C*), Joro 75$^+$ marrow cells (*D*), or PBS only (*A*) 8 wk before analysis.

but no Joro$^+$ cells were found in the medulla (data not shown).

The analysis of tissue sections from thymus at 2 d post-irradiation showed the presence of significant numbers of Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells. These Joro$^+$ cells were mainly in the subcapsular area and in the outer cortex but not in the medulla, and they all exhibited a lymphoblastoid morphology. Between days 4 and 6 post-irradiation, the Joro$^+$ cells tended to localize in the mid and deep cortex. Thymuses obtained at day 8 post-irradiation contained fewer Joro$^+$ cells, which, like in the normal thymus, were in the subcapsular region and outer cortex. For the sake of brevity, Fig. 6 illustrates the results with some examples obtained with the Joro 37-5 antibody only.

Thus, the immunohistochemical analysis confirms and complements the information obtained by FM analysis in a separate experiment. Taken together, the results from these experiments indicate first that there are normally very few Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ lymphoid cells in the thymus of young C57BL/6 mice, and that they are preferentially in the subcapsular area and outer cortex, but not in the medulla. These Joro$^+$ cells most probably represent recent T cell progenitor migrants from the bone marrow. Second, in the experimental system used here, the Joro$^+$ cells seem to colonize the thymus of sublethally irradiated mice by entering via blood vessels through the subcapsular and outer cortex regions, where they proliferate and subsequently migrate to inner areas of the cortex. Most Joro$^+$ cells must

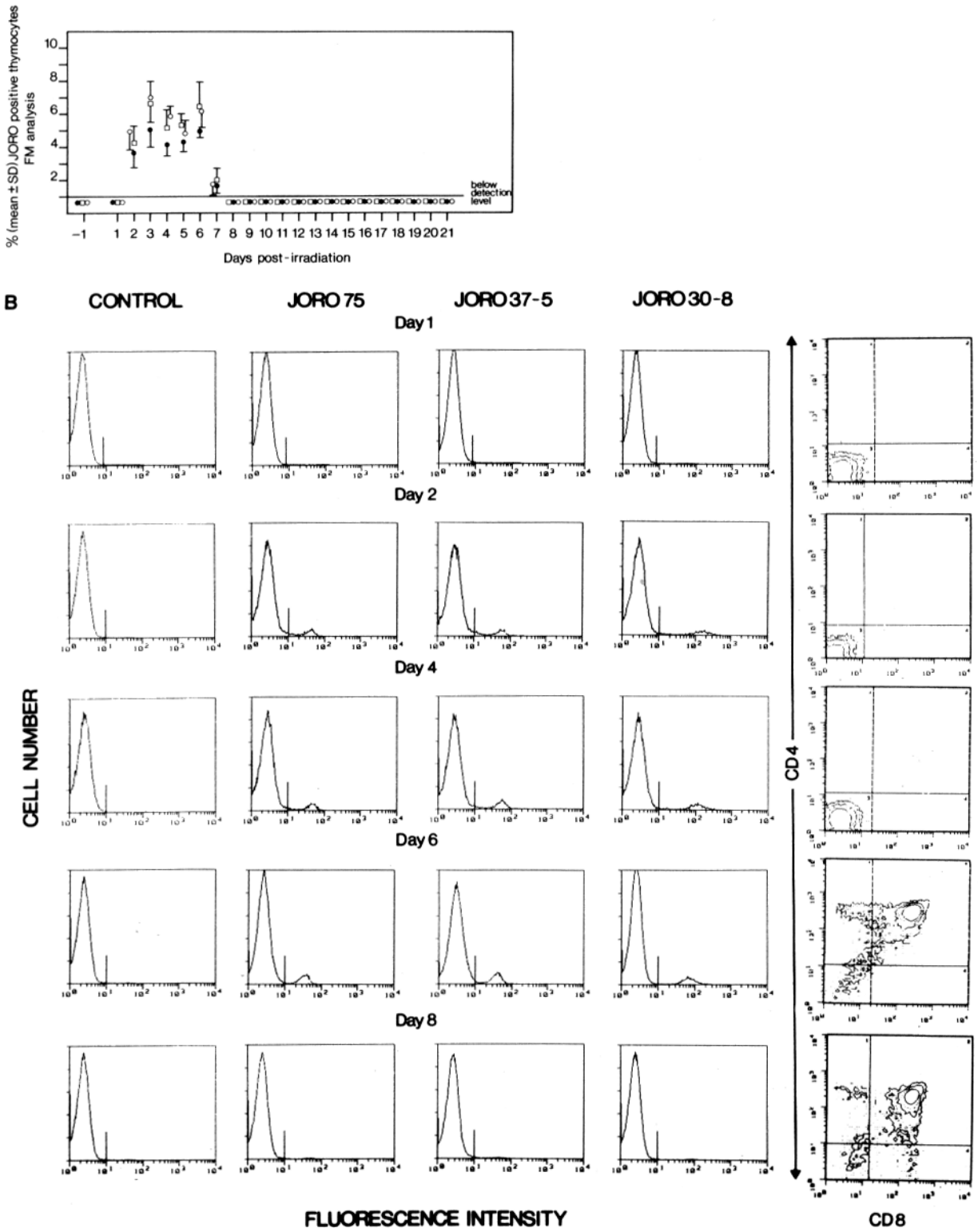


**Figure 5.** The presence of Joro 30-8$^+$, Joro 37-5$^+$, and Joro 75$^+$ cells in the thymocyte cell suspensions of C57BL/6 mice irradiated with 600 rad (140 rad/min) of $\gamma$ rays was assessed by FM analysis daily during 21 d using biotin-labeled Joro antibodies with FITC-streptavidin. Negative controls were cells stained with biotin-conjugated anti-rabbit IgG followed by FITC-streptavidin. The presence of CD4$^-$8$^+$, CD4$^+$8$^+$, and CD4$^+$8$^-$ thymocytes was assessed by two-color FM analysis. The data in *A* show the percentage (mean ± SD) of Joro$^+$ cells (●, Joro 30-8$^+$; ○, 37-5$^+$; □, Joro 75$^+$) detected in the three mice studied at each time point. *B* illustrates these results with some examples in the form of fluorescence histograms or contour plots.

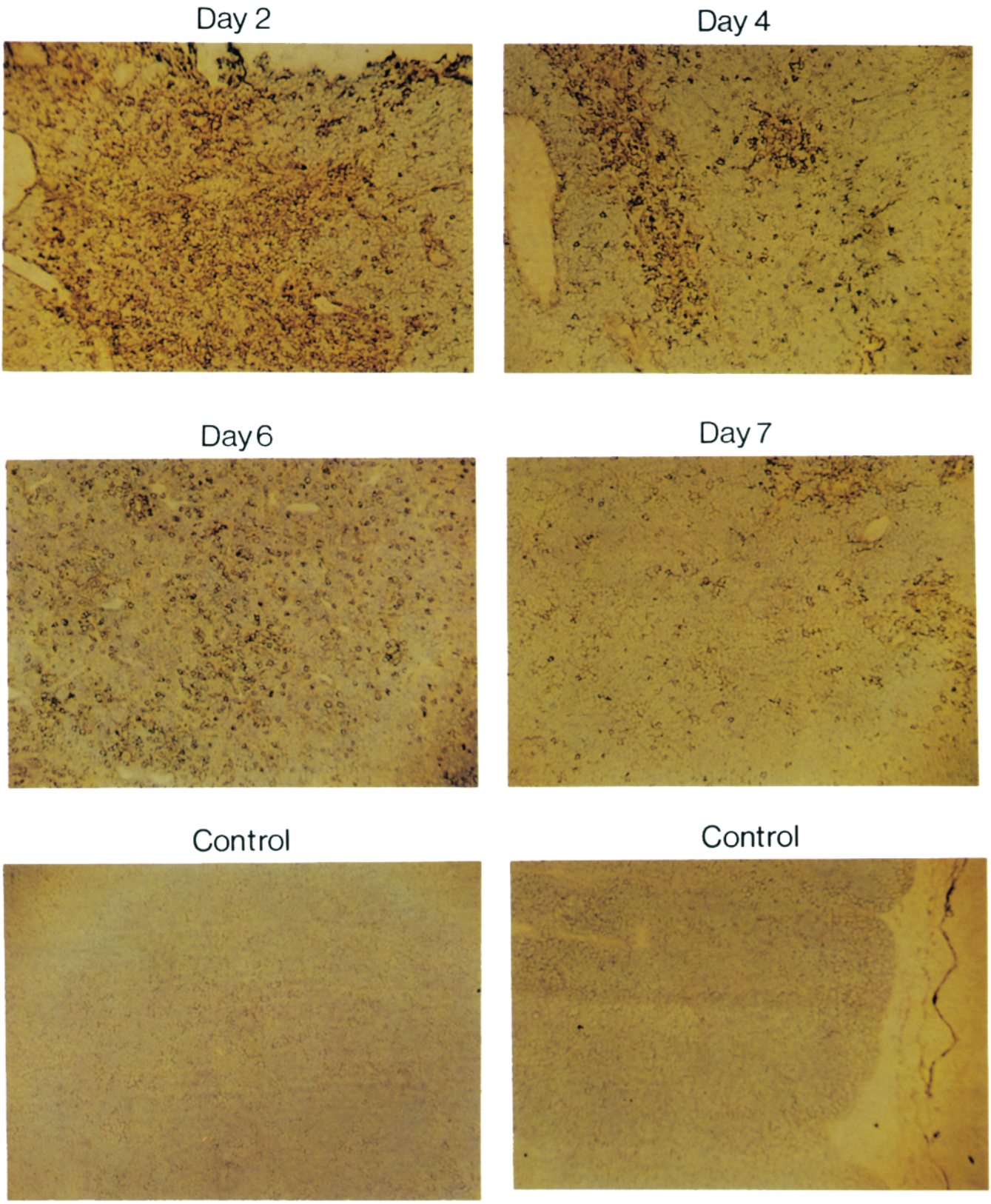


**Figure 6.** The presence and localization of Joro$^+$ cells in thymuses at various days post-irradiation of C57BL/6 mice was studied by immunohistochemistry. The results obtained with the Joro 37-5 mAb are only shown above for the sake of brevity and clarity. The same phenomena was found with the Joro 30-8 and Joro 37-5 mAbs.

differentiate into Joro$^-$ thymocytes within the cortex, since no Joro$^+$ cells are found in the medulla at any time, and since fewer Joro$^+$ cells were found at day 7–8 post-irradiation. While our results are of relevance to the process of thymus repopulation in the adult mice, it remains to be determined whether this process follows a similar pattern in the mouse embryo.

*Concluding Remarks.* The Joro mAbs described here appear to recognize cell surface determinants that are unique for early lymphocyte precursors, although we cannot exclude the possibility that they are also expressed by nonhemopoietic cells. Our results suggest that in bone marrow of young mice, cells expressing Joro 30-8 are very early hematopoietic precursor cells that are either committed to the lymphocyte lineage or that are even less differentiated pluripotent cells. Commitment to T cell differentiation seems to be associated with additional expression of Joro 37-5 and Joro 75.

It should be stressed that the experiments reported here were only designed to study hematopoietic cells that bind to the Joro 30-8, Joro 37-5, and Joro 75 mAbs; hence, they do not address the issue of whether the Joro antibodies detect all precursor cells for T lymphocytes. Experiments to define it are currently underway.

Recently it was shown that a Thy-1$^+$ Sca-1$^+$ population of marrow cells highly enriched for pluripotent stem cells give rise to thymocytes and peripheral T lymphocytes after intravenous transfer, and that after their direct transfer into the thymus of sublethally irradiated mice, a third of these Thy-1$^+$ Sca-1$^+$ marrow cells grew in this organ (11). The interpretation favored by the authors is that pluripotent stem cells as such migrate to the thymus, and that commitment to develop along the T cell pathway only takes place once the stem cell has entered this organ (11). Our findings do not necessarily argue against this scenario, but the present results, as well as our previous studies (14, 32), and those of other groups (33–35), do provide strong evidence for the existence of progenitor cells committed to the T cell lineage before migration to the thymus.

It is possible that both pro-T lymphocytes and pluripotent stem cells as such may colonize the thymus and generate T lymphocytes. However, because the thymus-repopulating activity of the Thy-1$^+$ Sca-1$^+$ marrow cell subset was assessed 14–28 d after their transfer, the possibility that Thy-1$^+$ Sca-1$^+$ stem cells (assuming that the isolated population comprises only pluripotent stem cells) first generated pro-T lymphocytes, which in turn migrated to the thymus, was not formally excluded (11). Actually, we have preliminary evidence that stem cells generate in vitro Joro$^+$ cells (J.C. Gutierrez and R. Palacios, unpublished observations). Also, the results in the study of Sprangrude et al. (11), showing that a third of a population of Thy-1$^+$ Sca-1$^+$ marrow cells (clearly highly enriched for pluripotent stem cells) grew in the thymus of sublethally irradiated mice after their intrathymic transfer (11), only documents that the thymic environment of irradiated mice can support proliferation of some Thy-1$^+$ Sca-1$^+$ marrow cells. It does not address the issue of whether or not pluripotent stem cells as such normally migrate to the thymus via physiological routes (i.e., via blood). Several types of T lineage cells (e.g., pro-T [13], CD4$^-$8$^-$ TCR/CD3$^-$ thymocytes [36], CD4$^-$8$^-$ TCR-$\alpha/\beta$–CD3$^+$ T cell lines (21), CD4$^+$8$^+$ thymocytes [37]) with or without the capacity to repopulate the thymus via physiological routes (after intravenous transfer) grow in the thymus after intrathymic injection. Thus, we think that direct evidence is still needed to accept that pluripotent stem cells as such normally migrate and colonize the thymus. This may be feasible to address when antibodies specific for pluripotent stem cells become available, and performing kinetic studies such as those used here to study the thymus repopulating potential of the Joro$^+$ cells.

The Joro 30-8, Joro 37-5, and Joro 75 mAbs together with recently developed in vitro systems for T cell differentiation (18) should facilitate the study of cellular and molecular events of prethymic and early intrathymic phases of T lymphocyte development. The specificity and development stage–restricted expression of the molecules recognized by these mAbs raise the possibility that they might function in early interactions of pro-T lymphocytes with a set of bone marrow stromal cells and thymic stromal components, perhaps as adhesion molecules or ligands involved in homing to the thymus. We shall now attempt to isolate cDNAs encoding the molecules recognized by the Joro mAbs as a complementary approach to determine their function.

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