Brief Definitive Report

Fetal Liver and Bone Marrow JORO 75 + Lymphocyte Progenitors Are Precursors of CD4+8 - TCR/CD3- Early Thymocytes
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Summary

We show here that cell sorter purified JORO 75 + lymphocyte progenitors from fetal liver or bone marrow of adult mice give rise in vitro to CD4+8 - T cell receptor (TCR)/CD3- early thymocytes and CD4+8 - TCR/CD3 + thymocyte subsets after coculture with the EH6 subcapsular thymic epithelial cell line, recombinant interleukin 7 (rIL-7) and F (supernatants from the FLS4.1 fetal liver stromal cell line). We find that in cultures that had additionally received rIL-2, CD4-8 + TCR/CD3 + cells were also generated. The results strongly suggest that fetal liver and marrow JORO 75 + lymphocyte progenitors are precursors to the early CD4+8 - TCR/CD3- intrathymic population previously identified in the adult mouse. The EH6 subcapsular thymic epithelial cell line should facilitate the study of the molecular events responsible for very early stages of T cell development including T lymphocyte lineage commitment.

Recent studies by Wu et al. (1, 2) report on the identification in the thymus of adult mice of a minute cell population that expressed low levels of CD4 on the cell membrane and could give rise to more mature thymocyte subsets after intrathymic transfer to irradiated animals. The authors referred to this subset as the earliest intrathymic precursor cells in the adult thymus or as the low CD4 precursor (1, 2). Previously we have described the characterization of the mAbs JORO 37-5 and JORO 75, which mark very early T cell progenitors present in the liver and thymus of the developing embryo and the bone marrow of young adult mice (3, 4). JORO + cells are very rare in the adult thymus, but they colonize this organ both in the embryo and in adult mice, expand, and give rise to α/β and 5/γ T lymphocytes (3, 4). The genes encoding JORO 37-5 and JORO 75 surface makers have no significant sequence similarity to any previously described gene that has been reported in the EMBL and the GenBank data bases (Palacios, R., manuscript in preparation).

We wished to determine whether a precursor-product relationship existed between JORO + fetal liver or adult bone marrow lymphocyte progenitors and the low CD4 thymocyte precursor cells. Neither we nor Shortman's group have found expression of JORO 75 or JORO 37-5 on the low CD4 thymocyte subset (our unpublished results). In the experiments described here, we tested the hypothesis that JORO + cells are precursors of low CD4 early thymocytes.

Materials and Methods

Mice. C57BL/6 young adult mice were bred and maintained in our animal facilities. Embryos were obtained from C57BL/6 timed matings. The day of detection of a vaginal plug was designated day 0.

Separation of Cells. Bone marrow mononuclear cells from young adult mice and fetal liver mononuclear cells from day 14-15 embryos were prepared as described before (3, 4). JORO + lymphocyte progenitors were isolated and purified by a negative selection step using a magnetic beads procedure followed by a positive selection step by cell sorting (Elite V; Coulter, Miami, FL) using biotin-labeled JORO 75 antibody and PE-streptavidin as described in detail before (5). Upon resanalysis, between 98.5 and 99.4% fetal liver cells and between 98.1 and 99.0% marrow cells were JORO +. The recovery was usually 0.3-0.5% from fetal liver cells and 0.1-0.4% from adult bone marrow. The cells were washed and resuspended in IMDM (GIBCO BRL, Gaithersburg, MD), 7.5% FCS (Hyclone Laboratories, Logan, UT), 2 mM l-glutamine, 5 x 10^-5 M 2-ME, and 50 μg/ml gentamycin (GIBCO BRL).

Cell Lines and Cytokines. The EH6 subcapsular thymic epithelial cell line was obtained from newborn (C57BL/6 x CBA)F1 mice by the procedure described earlier (6). These cells are morphologically and phenotypically different from cortical (e.g., ET, EA2) and medullary (e.g., EB3) thymic epithelial cells. They express cytokeratin and are positive for the NLDC-145 marker present on subcapsular epithelial and dendritic cells and negative for markers of cortical (ER-TR4, 4F1) or medullary (ER-TR5, IVC4) epithelial cells, fibroblasts (ER-TR7), myeloid cells (8C5, F4/80, Mac-1, BP-2, Mac-2, Mac-3), and lymphoid cells (JORO 75, JORO 37-5, B-220, IgM, CD4, CD8, CD3) (7-10). EH6 are nontransformed cells in that they do not form tumors in immunocompromised mice.

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rIL-7 was supernatants from J558 L/A2B2/44 myeloma cells transfected with mouse IL-7 cDNA (11); rIL-2 and rIL-3 were supernatants from X63 Ag8 myeloma cells transfected with mouse IL-2 or IL-3 cDNA (12). The cytokine preparations were tested for biological activity in proliferative assays as described (11). Supernatants collected from confluent cultures of the FLS4-1 fetal liver stromal cell line (13) will be referred to as F (for factor).

**Antibodies.** The following antibodies were used in FACSPur analysis: biotin-labeled JORO 75 or JORO37-5; FITC or biotin CD3 (145-2C11); FITC- or biotin-labeled TCR-γ/δ (H57/197) or TCR-β/γ (gL3); biotin CD44 (PgP-1, 145/2); and FITC or biotin Mac-1 (M1/60). FACSPur Analysis. FACSPur analysis was carried out as described before (3, 11) with the exception that a Coulter profile and Elite V machines were used. All stainings were done in the presence of purified rat IgG (250 μg/ml) and heat-inactivated hamster serum (15%) to prevent nonspecific Fc-receptor binding of labeled antibodies. Mononuclear cells from thymus, spleen, or fetal liver were used as positive controls as required. Cells stained with rat IgG or FITC or PE-streptavidin were used as negative control.

**Cell Culture Assay.** Six-well plates (Costar Corp., Cambridge, MA) containing monolayers of EH6 subcapsular thymic epithelial cells (50%–75% confluency) received fetal liver or adult bone marrow purified JORO 75+ cells (5 × 104 cells/well) and culture medium supplemented with rIL-7 (250–500 U/ml), F (10% vol/vol final concentration), and in some experiments rIL-2 (50–100 U/ml) or rIL-3 (100–200 U/ml) in a final volume of 2 ml/well. At day 5–6 of culture, 1 ml of fresh culture medium containing the cytokines indicated above was added to each well. Cells were harvested, washed, and tested by FACSPur analysis for the presence of several T cell lineage surface markers.

**Results and Discussion.**

In the course of experiments assessing the functional properties of the EH6 thymic subcapsular epithelial cell line, we found that these cells in the presence of rIL-7 and F (supernatants from the FLS4.1 fetal liver stromal line, which secrete Steel Factor and another factor that supports the formation of T cell progenitors and the proliferation of hematopoietic stem cells) supported the differentiation of purified JORO 75+ fetal liver cells along the T cell pathway. Indeed, JORO 75+ lymphocyte progenitors gave rise, under these culture conditions, to CD4+8+ TCR/CD3+ immature and to fewer CD4+8+ TCR/CD3- and CD4+8- TCR/CD3+ mature cells. No CD8+ or CD4+8+ cells were detected in these cultures. Only some of the cells in the cultures remained JORO 75+ or JORO37-5+, which is consistent with their differentiation into thymocytes, and most cells were PgP-1- (Fig. 1B and Table 1). JORO 75+ fetal liver cells before induction were CD4+8- TCR/CD3- PgP-1+ (Fig. 1A and data not shown). Similar results were obtained in the other three experiments we carried out (Table 1). These data show that JORO 75+ fetal liver progenitors are precursors to CD4+8+ TCR/CD3- immature and CD4+8- TCR/CD3+ mature thymocytes.

We thought it would be interesting to test whether JORO 75+ progenitors from the bone marrow of adult mice would exhibit a similar potential to that of JORO 75+ fetal liver progenitors. The results of such experiments are illustrated in the form of contour plots in Fig. 1 and are summarized in Table 1. Bone marrow–purified JORO 75+ progenitors cultured on EH6 subcapsular thymic epithelial cells, rIL-7 and F, also gave rise to CD4+8+ TCR/CD3+, and to fewer CD4+8- TCR/CD3+ and CD4+8- TCR/CD3+ cells (Fig. 1D). Purified JORO 75+ marrow progenitors before induction were CD4+8- TCR/CD3- PgP-1+ (Fig. 1C and data not shown). The CD3+ cells comprised both α/β+ and γ/δ+ cell lineages that were present at ratios that varied from experiment to experiment. Thus, JORO 75+ progenitors from either 14–15 day fetal liver or adult bone marrow are precursors to CD4+8+ TCR/CD3- immature and to more mature thymocyte subsets (see below).

Whereas the culture condition described above seems very useful to the study of early events after interactions between T cell progenitors and subcapsular thymic epithelium, it obviously cannot replace the thymic microenvironment. We have previously shown that there is functional heterogeneity of thymic epithelial cells in promoting differentiation of PRO-T cells into the different thymocyte subsets (6, 14), we were not surprised that not all thymocyte subsets developed in the cultures. Notwithstanding these considerations, we thought that the EH6 cell–based induction assay could also be used to test the effects of soluble cytokines on the developmental potential of JORO 75+ lymphocyte progenitors. To our surprise, we found that the addition of rIL-2 to the cultures promoted the generation of CD8+4- and some CD4+8+ thymocyte subsets, as well as the CD4+8- TCR/CD3-, CD4+8- TCR/CD3- and CD4+8- TCR/CD3+ populations that were seen in cultures incubated in the absence of rIL-2 (Fig. 1E and G; Table 1). In contrast, rIL-3 added to similar cultures increased the number of cells recovered at the end of the cultures, but did not significantly change the thymocyte populations generated by either fetal liver or adult bone marrow JORO 75+ progenitors in the absence of rIL-3 (Fig. 1F and H; Table 1). The results suggest that rIL-2 influences the developmental potential of JORO 75+ lymphocyte progenitors and promotes the formation of CD8+ thymocytes. These data are consistent with earlier studies (15–18) using quite different assays that have pinpointed a role for IL-2 in thymocyte development. They are apparently at odds with the information obtained in IL-2–deficient mutant mice, which showed quasi-normal thymocyte development (19). However, we believe that the latter could very well reflect the action of another cytokine (e.g., IL-4) that compensates for the deficiency of IL-2 in the mutant mice. Such compensatory or redundant cytokine function is cer-

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**Figure 1.** JORO 75+ progenitors from day 14–15 fetal liver (A, B, E, and F) or adult bone marrow (C, D, G, and H) were cultured on monolayers of EH6 subcapsular thymic epithelial cells in the presence of rIL-7 and F without (B and D) or with rIL-2 (E and G) or rIL-3 (F and H). A and C are from uninduced JORO 75+ progenitors from fetal liver (A) and adult marrow (C), respectively. After 6–8 d in culture, the presence of cells positive for the surface markers indicated was determined by two-color FACSPur analysis.
Table 1. Differentiation In Vitro of JORO 75+ T Cell Progenitors

| Precursor cells (induction assay) | Cell recovery | Percent positive cells FACS\(^\text{a}\) analysis |
|----------------------------------|---------------|------------------------------------------------|
|                                  | \(\times 10^6\) | CD4\(^-\) CD4\(^+\) CD4\(^-\) CD4\(^+\) CD4\(^-\) CD4\(^+\) JORO 75+ JORO 37-5+ PgP-1+ |
| JORO 75+ F. liver                |               |                                                  |
| EH6 + IL-7 + F                   |               |                                                  |
| Exp. 1                           | 2.4           | 20.3 3.4 0.1 0.2 1.7 0.3 26.8 28.4 99.2          |
| Exp. 2                           | 3.1           | 32.9 13.5 0.1 0.3 0.7 0.7 28.6 31.7 97.1          |
| Exp. 3                           | 2.6           | 15.4 4.6 0.1 0.2 1.6 0.2 24.4 24.9 99.5          |
| EH6 + IL-7 + F + IL-2            |               |                                                  |
| Exp. 1                           | 2.8           | 11.4 9.8 2.9 7.8 7.5 1.9 11.7 14.5 99.6          |
| Exp. 2                           | 3.9           | 17.7 21.4 5.8 12.7 8.2 3.1 10.0 10.9 98.5          |
| JORO 75+ adult bone marrow       |               |                                                  |
| EH6 + IL-7 + F                   |               |                                                  |
| Exp. 1                           | 4.7           | 13.5 2.0 0.2 0.3 1.7 0.5 35.7 37.4 98.6          |
| Exp. 2                           | 5.2           | 19.6 5.3 0.1 0.3 1.1 0.3 41.0 40.2 99.1          |
| EH6 + IL-7 + F + IL-2            |               |                                                  |
| Exp. 1                           | 2.5           | 9.9 14.5 3.6 8.9 4.2 1.6 12.7 14.9 97.5          |
| Exp. 2                           | 2.9           | 8.1 22.0 6.5 16.6 4.6 6.5 10.0 11.4 98.8          |

Cell sorter purified JORO 75+ progenitor cells were cocultured with EH6 subcapsular thymic epithelial cells, rIL-7, and F in the presence or absence of rIL-2 or rIL-3 at 37°C for 6–8 d. The presence of cells positive for the surface markers indicated above was assessed by two-color FACS\(^\text{a}\) analysis. Number of viable cells recovered per plate at the end of the culture. Input cells in all cultures were 3 \(\times 10^6\) per plate.

In conclusion, the experiments described here provide evidence that JORO 75+ lymphocyte progenitors in the fetal liver and in the adult bone marrow give rise in vitro to CD4\(^-\)8\(^-\) TCR/CD3\(^-\) early thymocytes. The EH6 subcapsular thymic epithelial cell line should help us to elucidate the molecular events responsible for very early stages of T cell development, including T lymphocyte lineage commitment and determine the role of cytokines in such a process.

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References

1. Wu, L., R. Scollay, M. Egerton, M. Pearse, G. Spangrude, and K. Shortman. 1991. CD4 expressed on earliest T lineage precursor cells in the adult murine thymus. *Nature (Lond.)* 349:71.

2. Wu, L., M. Antica, G.R. Johnson, R. Scollay, and K. Shortman. 1991. Developmental potential of the earliest precursor cells from adult mouse thymus. *J. Exp. Med.* 174:1617.

3. Palacios, R., J. Samaridis, D. Thorpe, and T. Leu. 1990. Identification and characterization of pro-T lymphocytes and lineage uncommitted lymphocyte precursors from mice with three novel surface markers. *J. Exp. Med.* 172:219.

4. Palacios, R., and J. Samaridis. 1991. Thymus colonization in the developing mouse embryo. *Eur. J. Immunol.* 21:109.

5. Palacios, R., and J. Samaridis. 1993. Bone marrow clones representing an intermediate stage of development between hematopoietic stem cells and pro-T lymphocyte or pro-B lymphocyte progenitors. *Blood.* 81:1222.

6. Palacios, R., J. Samaridis, S. Studer, and J. Pelkonen. 1989. Thymic epithelial cells induce in vitro differentiation of pro-T lymphocyte clones into TCR αβ/T3+ and TCR γδ/T3+ cells. *EMBO (Eur. Mol. Biol. Organ.)* J. 8:4053.

7. van Ewijk, W. 1988. Cell surface topography of thymic microenvironments. *Lab Invest.* 59:579.

8. van Vliet, E., M. Melis, and W. van Ewijk. 1984. Monoclonal antibodies to stromal cell types in the mouse thymus. *Eur. J. Immunol.* 14:524.

9. Lampert, I., and M.A. Ritter. 1990. The origin of the diverse epithelial cells of the thymus: is there a common stem cell? *Thymus Update.* 1:5.

10. van Ewijk, W. 1991. T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol.* 9:591.

11. Samaridis, J., G. Casorati, A. Traunecker, A. Iglesias, J. Gutierrez, U. Müller, and R. Palacios. 1991. Development of lymphocytes in interleukin-7 transgenic mice. *Eur. J. Immunol.* 21:453.

12. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete large quantities of interleukins 2, 3, 4, and 5 using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97.

13. Palacios, R., and J. Samaridis. 1992. Fetal liver pro-B and pre-B lymphocyte clones. Expression of lymphoid specific genes, surface markers, growth requirements, colonization of the bone marrow, and generation of B lymphocytes in vivo and in vitro. *Mol. Cell. Biol.* 12:518.

14. Gutierrez, J., and R. Palacios. 1991. Heterogeneity of thymic epithelial cells in promoting T-lymphocyte differentiation in vivo. *Proc. Natl. Acad. Sci. USA.* 88:642.

15. Jenkinson, E., L. Kingston, and J.J. Owen. 1987. Importance of interleukin 2-receptors in intrathymic generation of cells expressing T-cell receptors. *Nature (Lond.)* 329:160.

16. Möller, G., editor. 1988. T-cell precursors. *Immunol. Rev.* 104:5–250.

17. Shortman, K. 1992. Cellular aspects of early T-cell development. *Curr. Opin. Immunol.* 4:140.

18. Waanders, G., and R.L. Boyd. 1990. The effects of interleukin 2 on early and late thymocyte differentiation in fetal thymic organ cultures. *Int. Immunol.* 2:4671.

19. Schorle, H., T. Holtschke, T. Hüning, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin 2 deficient by gene targeting. *Nature (Lond.)* 352:621.