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Rat CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ Thymocytes

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T cells differentiate and mature in the thymus. Therefore, the thymus has been considered a central lymphoid organ for cellular immunity (1, 2). It is important to elucidate events in the thymus when studying cellular immunity.

Thymocytes are divided into four major populations, CD4$^-$ CD8$^-$, CD4$^+$ CD8$^+$, CD4$^+$ CD8$^-$ and CD4$^-$ CD8$^+$ thymocytes, on the basis of expression of the cell surface antigens CD4 and CD8 (3-5). The CD4$^-$ CD8$^-$ thymocytes, which are precursor cells from bone marrow and the most immature, give rise to the CD4$^+$ CD8$^+$ cells, which account for the majority of thymocytes. At this stage, thymocytes are subjected to positive and negative selcction (6-8) and develop into the mature CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ cells. This differentiation sequence results in the production of mature, self-tolerant, self-major histocompatibility complex (MHC)-restricted T cells. This maturation process has recently been further examined with regard to other cell surface antigens, including CD3 (9-11), T cell antigen receptor (TCR) $\alpha\beta$ (12-15), the interleukin-2 receptor (CD25) (14, 16), phagocytic glycoprotein-1 (pgp-1, CD44) (14) and heat-stable antigen (HSA) (14, 17), and has been extensively reviewed elsewhere (18-20). However details of this differentiation sequence remain obscure.

CD4$^-$ CD8$^-$ TCR$\alpha\beta^+$ cells can be detected in bone marrow, thymus, spleen, lymph nodes and peripheral blood. These cells can be unambiguously classified as mature T cells by their phenotype; for example, they are CD3$^+$, CD5$^+$, CD25$^+$ (after stimulation), and HSA$^-$ (5, 21-25). Because autoreactive cells are present among the CD4$^-$ CD8$^-$ TCR$\alpha\beta^+$ cells, it has been suggested that they play a role in autoimmune disease (26). The CD4$^-$ CD8$^-$ TCR$\alpha\beta^+$ cells develop in the thymus and show a delayed appearance in thymic organ culture (21). Proliferation of these cells is induced through the CD3-dependent activation pathway in the presence of interleukin-1 (5, 21, 22). Furthermore, a selective increase in functionally mature CD4$^-$ CD8$^-$ TCR$\alpha\beta^+$ thymocytes occurs after injection of monoclonal antibodies to CD2 (27). Recently, an increase in these cells was observed during recovery from thymus atrophy induced by di-n-butyltin dichloride (28).

We previously analyzed the effects of cyclosporin A (29), FK506 (30-35), glucocorticoid (GC) treatnlent (35, 36) and irradiation (32, 36) on rat thymus, with the usage of specific monoclonal antibodies, the immunoperoxidase technique and flow cytofluorometry. In this report we present the

existence of the CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ thymocytes in normal adult rats, and the possible implications of the differentiation and maturation of this subpopulation are discussed.

Three eperimental animals were prepared. In brief, adult Lewis (RT-1^1) rats were irradiated (600 rads/body), received dexamethasone (0.1mg, by single administration), or received FK506 (1mg/kg of body mass) for 7 days. Rats were killed and the thymi were removed from three rats for flow cytofluorornetry on days 0 (before treatment), 3, 5, 7 and 14 after irradiatin, on days 0, 3, 5, 7 and 10 after GC treatmen, and on days 7 and 14 after FK506 treatment. The cell-surface antigens CD4, CD8 and TCR$\alpha\beta$ of rat thymocytes were on each time analyzed by three-color flow cytofluorometry. These materials and methods were described peviously in detail (35).

Firstly, we have studied the expression of TCR$\alpha\beta$ in rat CD4$^-$ CD8$^-$ thymocytes during thymic reconstitution after irradiation or GC treatment. Irradiation induced a marked decrease in the percentage of CD4$^+$ CD8$^+$ thymocytes on day 3, when a maximum decrease in thymus mass and total thymocyte number was observed, in parallel with a relative increase in the percentages of CD4$^-$ CD8$^-$, CD4$^-$ CD8$^-$ and CD4$^-$ CD8$^+$ thymocytes. A rapid thymic reconstitution was observed thereafter and the percentage of CD4$^+$ CD8$^+$ thymocytes increased rapidly. The mean percentage of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells was decreased on days 3 and 5 but markedly increased on days 7 and 14 after irradiation (table 1). Similar changes were observed during thymic reconstitution after a single treatment with GC (data not shown). These results indicate that some CD4$^-$ CD8$^-$ cells appeared to increase their expression of TCR$\alpha\beta$ gradually, developing into mature CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells, although most CD4$^-$ CD8$^-$ cells developed into mature CD4$^+$ CD8$^-$ or CD4$^-$ CD8$^+$ cells via the CD4$^+$ CD8$^+$

Table 1. Thymus mass, total thymocyte number, thymocyte populations and the CD4$^-$8$^-$ TCR$\alpha\beta^{high}$ and CD4$^-$8$^-$ TCR$\alpha\beta^{low}$ subpopulations after irradiation.

Time after irradiation (days)	Thymus mass (mg)	Total number of thymocytes ($\times10^8$)	CD4 and CD8 expression (%)*				CD4$^-$8$^-$ TCR$\alpha\beta^{high}$(%)**	CD4$^-$8$^-$ TCR$\alpha\beta^{low}$(%)**
			CD4$^-$8$^-$	CD4$^+$8$^+$	CD4$^+$8$^-$	CD4$^-$8$^+$		
0	417.0±20	10.7±1.6	4.2±2.2	82.4±1.4	7.2±0.5	4.2±0.8	12.6±5.4	5.9±4.4
3	88.0±10	0.04±0.01	30.9±12	11.0±1.8	33.2±13	25.0±1.2	4.5±1.5	26.4±20
5	113.3±17	0.32±0.03	3.7±0.3	88.9±0.4	2.9±0.5	4.5±0.5	4.1±0.9	10.1±0.3
7	160.7±41	1.78±0.63	1.1±0.2	85.6±2.0	8.9±0.4	4.4±1.5	28.6±10	5.3±3.1
14	286.7±17	4.82±0.12	3.5±1.3	76.2±2.8	16.0±1.4	4.3±0.4	27.0±1.1	32.1±4.9

Values are means ± S.D. (n=3)
*Values represent a percentage of total thymocytes.
**Values represent a percentage of CD4$^-$8$^-$ cells.

Table 2. Thymus mass, total thymocyte number, thymocyte populations and the CD4$^-$8$^-$ TCR$\alpha\beta^{high}$ subpopulation after FK506 treatment.

Time after FK506 treatment (days)	Thymus mass (mg)	Total number of thymocytes ($\times10^8$)	CD4 and CD8 expression (%)*				CD4$^-$8$^-$ TCR$\alpha\beta^{high}$(%)**
			CD4$^-$8$^-$	CD4$^+$8$^+$	CD4$^+$8$^-$	CD4$^-$8$^+$	
0	421.5±10	9.75±2.5	4.2±2.2	82.4±1.4	7.2±0.5	4.2±0.8	12.6±5.4
7	310.3±34	7.78±2.2	2.7±0.2	93.5±0.8	1.4±0.3	1.9±0.2	0.25±0.08
14	320.0±43	9.25±0.8	2.2±0.2	95.8±0.1	0.6±0.03	1.4±0.07	1.1±0.11

Values are means ± S.D. (n=3)
*Values represent a percentage of total thymocytes.
**Values represent a percentage of CD4$^-$8$^-$ cells.

stage.

Secondary, we examined the effect of FK506 on CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells. Continuous treatment with FK506 for 7 days induced a marked decrease not only in CD4$^+$ CD8$^-$ TCR$\alpha\beta^{high}$ and CD4$^-$ CD8$^+$ TCR$\alpha\beta^{high}$ cells but also in CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells (table 2). Similarly, Takahama et al. (37) showed that cyclosporin A induced maturation arrest of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells. FK506 is a macrolide immunosuppressive agent that is isolated from the soil fungus *Streptomyces tukubaensis*. The immuno-suppressive action of FK506 appears to result from interference with the TCR signal transduction pathway; specially, from inhibition of the Ca^{2+}- and calmodulin-dependent phosphoprotein phosphatase calcineurin (38, 39), a key signaling enzyme in T cell activation (40). It has been previously shown that FK506 markedly reduces the size of the thymic medulla, especially the MHC class II-positive area (31-33, 41). MHC class II-positive thymic stromal cells, particularly the epithelial cells, are important for T cell maturation, differentiation, acquistiuon of MHC restriction (42). Our demonstration that FK506 decreased the percentage of mature thymocytes (CD4$^+$ CD8$^-$ TCR$\alpha\beta^{high}$ and CD4$^-$ CD8$^+$ TCR$\alpha\beta^{high}$ cells) is therefore consistent with these previous observations. The CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells are thought to escape the clonal deletion (43), which may be induced through the intercellular communications between thymocytes and MHC class II-positive intrathymic marrow-derived macrophages and dendritic cells (42). However, in our experiments, FK506 markedly decreased the percentage of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells. Furthermore, other researches have demonstrated that splenic CD4$^-$ CD8$^-$ TCR$\alpha\beta^+$ cells from normal mice show MHC class II- restricted autoreactivity in vitro (23, 24). We therefore suggest that MHC class II-positive thymic epithelial cells, which are impaired by FK506 treatment, may be important for maturation and differentiation not only of CD4$^+$ CD8$^-$ TCR$\alpha\beta^{high}$ and CD4$^-$ CD8$^+$ TCR$\alpha\beta^{high}$ cells but also of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells. It has been previously shown that FK506 also reduces the OX6$^+$, PKK-1$^+$ (α-cytokeratin), AB-1040$^+$ (type IV collagen) and AB-1220$^+$ (laminin) areas in the thymic cortex (31). The maturation and differentiation of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells may thus be dependent on one or more of these cortical thymic cell types. Furthermore, Tiefenthaler et al. (27) recently showed that CD2 stimulation resulted in an increase in CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ thymocytes in rats. In mice, however, maturation of the CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells appeared to occur without detectable CD2 (44). These conflict observations may be attributable to the fact that the natural ligands of CD2 molecule in mice and rats are CD48 and CD58, respectively (45-47). CD58$^+$ thymic epithelial cells may thus also be affected by FK506.

Cell surface antigens; the interleukin-2 receptor (CD25), phagocytic glycoprotein-1 (pgp -1, CD44), heat-stable antigen (HSA) and others can further subdivide thymocyte population (14, 16, 17, 18-20). It will thus be of interest to analyze their cell surface antigens on the CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells in normal rat thymus or after irradiation, GC treatment or FK506 treatment. Furthermore, approximately 50% of CD4$^-$ CD8$^-$ TCR$\alpha\beta^{high}$ cells express TCRs containing Vβ8 (21, 22). It will also be of interest to determine the Vβ expression.

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References

1. Osoba, D. and Miller, J.F.A.P. : Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. *Nature* **199**: 653-654, 1963.
2. Miller, J.F.A.P. : Immunological function of the thymus. *Lancet*. i: 748-749, 1961.
3. Mason, D.W., Arthur, R.P., Dallman, M.J., Green, J.R., Spickett, G.P. and Thomas, M. L. : Functions of rat T-lymphocyte subsets isolated by means of monoclonal antibodies. *Immunol. Rev.* **74**: 57-82, 1983.
4. Scollay, R., Bartlett, P. and Shortman, K.

: T cell development in the adult murine thymus. Changes on the expression of the cell surface antigens Ly2, L3T4 and B2A2 during development from early precursor cells to emigrants. *Immunol. Rev.* **82**: 79-103, 1984.

5. Crispe, L.N., Moore, M.W., Husman, L.A., Smith, L., Bevan, M.J. and Shimonkevitz, R.P.: Differentiation potential of subsets of $CD4^-$ $CD8^-$ thymocytes. *Nature* **329**: 336-339, 1987.

6. Blackman, M., Kappler, J. and Marrack, P. : The role of the T cell receptor in positive and negative selection of developing T cells. *Science* **248**: 1335-1341, 1990.

7. Ramsdell, F. and Fowlkes, B.J. : Clonal deletion versus clonal anergy: The role of the thymus in inducing self tolerance. *Science* **248**: 1342-1348, 1990.

8. von Boehmer, H. : Thymic selection: a matter of life and death. Immunol. Today **13**: 454-458, 1992.

9. MacDonald, H.R., Budd, R.C. and Hower, C. : A $CD3^-$ subset of $CD4^-$ 8^+ thymocytes: a rapidly cycling intermediate in the generation of $CD4^+$ 8^+ cells. *Eur. J. Immunol.* **18**: 519-523, 1988.

10. Hugo, P., Waanders, G.A., Scollay, R., Shortman, K. and Boyd R.L. : Ontogeny of a novel $CD4^+$ $CD8^-$ $CD3^-$ thymocyte subpopulation: a comparison with $CD4^-$ $CD8^+$ $CD3^-$ thymocytes. *Int. Immunol.* **2**: 209-218, 1990.

11. Komatsu, K., Yoshikai, Y., Mori, Y., Asano, T., Ando, T. and Nomoto, K.: Two differential pathway from double-negative to double-positive thymocytes. *Immunology* **72**: 20-26, 1991.

12. Hünig, T., Wallny, H.J., Hartly, J.K., Lawetzky, A. and Tiefenthaler G. : A monoclonal antibody to a constant determinant of the T cell antigen receptor that induces T cell activation. *J. Exp. Med.* **169**: 73-86, 1989.

13. Hünig, T. and Mitnacht, R. : T cell receptor-mediated selection of functional rat CD8 T cells from defined immature thymocyte precursors in short-term suspension culture. *J. Exp. Med.* **173**: 561-568, 1991.

14. Pearse, M., Wu, L., Egerton, M., Wilson, A., Shortman, K. and Scollay, R. : A murine early thymocyte developmental sequence is marked by transient expression of the interleukin 2 receptor. *Proc. Natl. Acad. Sci. USA.* **86**: 1614-1618, 1989.

15. Nikolić-Žugić, J., Moore, M.W. and Bevan, M.J. : Characterization of subset of immature thymocytes which can undergo rapid in vitro differentiation. *Eur. J. Immunol.* **19**: 649-653, 1989.

16. Shimonkevitz, R.P., Husman, L.A., Bevan, M.J. and Crispe, I.N.: Transient expression of IL-2 receptor precedes the differentiation of immature thymocytes. *Nature* **329**: 157-159. 1987.

17. Shortman, K. and Wilson, A. : Immature $CD4^-$ $CD8^+$ murine thymocytes. *Cell. Immunol.* **113**: 462-477, 1988.

18. Nikolić-Žugić, J . : Phenotypic and functional stages in the intrathymic development of $\alpha\beta$ T cells. *Immunol. Today* **12**: 65-70, 1991.

19. Boyd, R.L. and Hugo, P. : Towards an integrated view of thymopoiesis. *Immunol. Today* **12**: 71-79, 1991.

20. Godfrey, D.I. and Zlontik, A. : Control points in early T-cell development. *Immunol. Today* **14**: 547-553, 1993.

21. Fowlkes, B.J., Kruisbeek, A., Ton-That, H., Weston, M.A., Coligan, J.E., Schwartz, R.H. and Pardoll, D.M.: A novel population of T-cell receptor $\alpha\beta$-bearing thymocytes which predominantly expresses a single Vβ gene family. *Nature* **329**: 251-254, 1987.

22. Budd, R.C., Miescher, G.G., Howe. R.C., Lees, R.K., Bron, C. and MacDonald, H.R. : Developmentally regulated expression of T cell receptor β chain variable domains in immature thymocytes. *J. Exp. Med.* **166**: 577-582, 1987.

23. de Talance, A., Regnier, D., Spinelli, S., Morisset, J. and Seman, M. : Origin of autoreactive T helper cells. I. Characterization of Thy-1^+, Lyt^-, $L3T4^-$ precursors in the spleen of normal mice. *J. Immunol.* **137**: 1101-1108, 1986.

24. Reimann, J., Bellan, A. and Conrad, P. : Development of autoreactive $L3T4^+$ T cells from double-negative ($L3T4^-$/Ly-2^-) Thy-1^+ spleen cells of normal mice. *Eur. J. Immunol.* **18**: 989-999, 1988.

25. Reimann, J. : Double-negative ($CD4^-$ $CD8^-$), TCR $\alpha\beta^-$ expressing, peripheral T

cells. *Scand. J. Immunol.* **34**: 679-688, 1991.
26. Singer, P. A. and Theofilopoulos, A. N. : T-cell receptor Vβ repertoire expression in murine models of SLE. *Immunol. Rev.* **118**: 103-127, 1990.
27. Tiefenthaler, G., Hanke, T. and Hünig, T. : Maturation of CD4⁻ CD8⁻ α/β TCR⁺ T cells induced by CD2-stimulation in vivo and in vitro. *Int Immunol.* **4**: 825-829, 1992.
28. Pieters, R.H.H., Bol, M., Lam, B.W., Seinen, W., Bloksma, N. and Penninks, A. H. : Recovery from chemically induced thymus atrophy starts with CD4⁻ CD8⁻ $CD2^{high}$ TCR $\alpha\beta^{-/low}$ thymocytes and results in an increased formation of CD4⁻ CD8⁻ TCR $\alpha\beta^{high}$ thymocytes. *Immunology* **78**: 616-622, 1993.
29. Tanaka, M., Shinohara. K., Fukumoto, T., Tanaka, H. and Kaneko, T. : Effect of Cyclosporin A on rat thymus : time course analysis by immunoperoxidase technique and flow cytofluorometry. *Clin. Exp. Immunol.* **72**: 216-221, 1988.
30. Takai, K., Jojima. K., Sakatoku, J. and Fukumoto, T. : Effects of FK506 on rat thymus: time-course analysis by immunoperoxidase technique and flow cytofluorometry. *Clin. Exp. Immunol.* **82**: 445 -449, 1990.
31. Takai, K., Tokuda, N., Sawada, T., Fujikura, Y., Jojima, K., Sakatoku, J. and Fukumoto T. : Effects of FK506 on rat epithelial cells; immunohistochemical study. *Thymus* **19**: 207-217, 1992.
32. Konishi, M., Takai, K., Jojima, K., Fujikura, Y., Naito, K. and Fukumoto, T. : Effects of FK506 on surface antigen expression by generating thymocytes after sublethal irradiation in the rat. *Thymus* **23**: 53-66, 1994.
33. Takai, K., Tsuchida, M., Konishi, M., Tokuda, N., Fujikura, Y., Jojima, K. and Fukumoto, T. : Immunosuppressive effects of FK506 on rat lymphoid organs. *Transplant. Proc.* **23**: 2964-2966, 1991.
34. Tsuchida, M., Takai, K., Jojima, K., Naito, K., Fujikura, Y. and Fukumoto, T. : The effects of FK506 and dexamethasone on rat thymocyte differentiation. *Ther. Immunol.* **1**: 135-141, 1994.
35. Tsuchida, M., Konishi, M., Takai, K., Naito, Y., Fujikura, Y. and Fukumoto, T.: Effects of irradiation, glucocorticoid and FK506 on cell-surface antigen expression by rat thymocytes: a three-colour flow cytofluorometric analysis. *Immunology* **83**: 469-475, 1994.
36. Tsuchida M., Konishi, M., Jojima, K., Naito, K., Fujikura, Y. and Fukumoto, T.: Analysis of cell surface antigens on glucocorticoid-treated rat thymocytes with monoclonal antibodies. *Immunol. Lett.* **39**: 209-217, 1994.
37. Takahama, Y., Kosugi, A. and Singer, A.: Phenotype, ontogeny, and repertoire of CD4⁻ CD8⁻ T cell receptor $\alpha\beta^{+}$ thymocytes . Variable influence of self- antigens on T cell receptor Vβ usage. *J. Immunol.* **146**: 1134-1141, 1991.
38. Liu, J., Farmer, Jr. J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. : Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66**: 807-815, 1991.
39. O'keefe, S.J., Tamura, J., Kincaaid, R.L., Tocci, M.J. and O'neill, E.A. : FK-506- and CsA-sensitive activation of the interleukin -2 promoter by calcineurin. *Nature* **357**: 692 -694, 1992.
40. Clipstone, N.A. and Crabtree, G.R. : Identification of calcineurin as a key signaling enzyme in T-lymphocyte activation. *Nature* **357**: 695-697, 1992.
41. Pugh-Humphreys, R.G.P., Ross, C.S.K. and Thomson, A.W.: The influence of FK- 506 on the thymus: an immunophenotypic and structural analysis. *Immunology* **70**: 398 -404, 1990.
42. Sprent, J., Lo, D., Gao, E-K. and Ron, Y.: T cell-selcction in the thymus. *Immunol. Rev.* **101**: 172-190, 1988.
43. Teh, H.-S., Kishi, H., Scott, B. and von Boehmer, H. : Deletion of autospecific T cells in T cell receptor (TCR) transgenic mice spares cells with normal TCR levels and low levels of CD8 molecules. *J. Exp. Med.* **169**: 795-806, 1989.
44. Gotlieb, W.H., Durum, S.K., Gregorio, T. A. and Mathieson, B.J. : Selective stimulation of thymocytes precursors mediated by specific cytokines. Different CD3⁺ subsets are generated by IL-1 versus IL-2. *J. Immunol.* **146**: 2262-2271, 1991.
45. Kato, K., Koyanagi, M., Okada, H., Takanashi, T., Wong, Y.W., Barclay, A.N.,

Williams, A.F., Okumura, K. and Yagita, H. : CD48 is a counter-receptor for mouse CD2 and involved in T cell activation. *J. Exp. Med.* **176**: 1241-1249, 1992.
46. Denning, S.M., Tuck, D.A., Vollger, L. W., Springer, T.A., Singer, K.H. and Haynes, B. F. : Monoclonal antibodies to CD2 and lymphocyte function-associated antigen 3 inhibit human thymic epithelial cell-dependent mature thymocyte activation. *J. Immunol.* **139**: 2573-2578, 1987.
47. Clark, S.J., Law. D.A., Paterson, D.J., Puklavec, M. and Williams, A.F. : Activation of rat lymphocytes by anti-CD2 monoclonal antibodies . *J. Exp.* Med. **167**: 1861 -1872, 1988.