## *Brief Definitive Report*

# ESTABLISHMENT OF HUMAN DOUBLE-POSITIVE THYMOCYTE CLONES

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CD4 and CD8 are accessory molecules expressed on distinct subsets of peripheral T lymphocytes that recognize antigens in association with class II and class I MHC molecules, respectively. Although peripheral T cell pool contains only single-positive (SP) CD4 or CD8 cells, a large subset of thymocytes are positive for both molecules (1). These double-positive (DP) cells, which differentiate in the thymus to membrane expression of TCR (2–4), have been the subject of much debate. The failure to expand such cells in vivo or in vitro was taken as evidence that they represent a "dead-end" population (5, 6). However, recent evidence suggests that DP cells may be precursors of mature SP lymphocytes (7–10).

We report here the establishment and characterization of DP clones from human thymus. These cells show a stable DP phenotype, express a functional CD3-TCR complex, and give rise in vitro to SP variants.

## Materials and Methods

*Monoclonal Antibodies.* The following mAbs were used: Leu 3a and Leu 3b (anti-CD4), Leu 2a (anti-CD8), 55.2 (anti-CD2), Leu19, Leu 9 (anti-CD7), Leu 1 (anti-CD5) (Becton Dickinson & Co., Mountain View, CA), OKT3 (anti-CD3, American Type Culture Collection [ATCC], Rockville, MA), 25.3 (anti-CD11a) and BL5 (anti-CD18) (Immunotech, Luminy, France), δ1 (anti-TCR δ chain), WT31 (which recognizes a CD3 epitope present on α/β cells), BMA032 (anti-TCR-α/β), Na1/34, WM25, and 10C3 (anti-CD1a,b,c). Anti-CD4 and anti-CD8 were direct labeled and the other mAbs were used unlabeled and detected with FITC-conjugated goat anti-mouse Ig (Becton Dickinson & Co., Mountain View, CA).

*Cloning of Thymic Cells.* Thymic and peripheral blood samples were obtained from three patients (1, 1, and 4 yr old) undergoing heart surgery. Thymus cell suspensions were purified on Ficoll gradients and stored frozen in 10% DMSO. After thawing, living cells were recovered through a Percoll cushion (1.078 density), washed twice, and doubly labeled with anti-CD4 and anti-CD8 mAbs. Double-negative (DN), DP, CD4, and CD8 SP cells were sorted using a FACS440 sorter (Becton Dickinson & Co.). Cells were cloned by limiting dilution in Terasaki plates (Nunc, Roskilde, Denmark) in 20 μl RPMI 1640 medium (Gibco Laboratories, Grand Island, NY), supplemented with 5% human serum, 2 mM L-glutamine, 1% nonessential aminoacids, 1 mM sodium pyruvate, 50 μg/ml Kanamycin (Gibco Laboratories), in the presence of 1 μg/ml PHA (Wellcome, Dartford, UK), 100 U/ml human rIL-2 (Hoffman-La Roche, Nutley, NJ) and 5 × $10^5$/ml 3,000 rad irradiated allogeneic PBMC as feeders. After 10 d, positive wells were scored and cells were further restimulated and expanded as above.

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*Immunofluorescence.* Cells were incubated for 30 min with mAbs at 4°C in PBS containing 0.01% azide and 0.5% BSA, and analyzed in a FACScan flow cytometer (Becton Dickinson & Co.) with logarithmic amplification using FACScan software. Live gates were set by a combination of low-angle forward and sideways light scatter. Markers were set against the plots of control samples labeled with either anti-CD4 or anti-CD8 (in double fluorescence), or second reagent alone (in single fluorescence).

*mAb-facilitated Cytolysis.* $2 \times 10^3$ $FcR^+$ P815 mastocytoma cells labeled with $^{51}Cr$ were incubated in 200 μl RPMI/10% FCS with effector cells in the presence of 1 μg/ml OKT3 or BMA032. Specific $^{51}Cr$ release was determined in a standard 4-h assay.

## Results and Discussion

*Establishment of Double-Positive Clones.* Human thymocytes were doubly stained with anti-CD4 and anti-CD8 mAbs, sorted, and cloned by limiting dilution. Clones from all four populations (DP, CD4, or CD8 SP and DN) were established. In five experiments and from three different thymuses, DP cells cloned with an efficiency of 1–3%. The cloning efficiency of SP CD4 or CD8 cells was 10–30%, and that of DN cells was 0.5–8%. SP cells from peripheral blood of the same donors cloned with an efficiency of 10–30%, which is also rather low, and is likely to be due to extensive manipulation of the frozen cells. The poor cloning efficiency of DP cells could be due either to intrinsic fragility or lower responsiveness to triggering stimuli (11). We were, however, surprised to be able to clone these populations, in view of the reported failures to expand DP cells in vitro or in vivo in the mouse (5, 6), and of the fact that human DP cells have been maintained in culture only for short periods (12).

All clones were analyzed for CD4, CD8, and TCR expression (Table I). 80% of the clones from the sorted DP subset expressed both CD4 and CD8, while 20% were SP. These could represent either DP cells which became SP in vitro, or a contaminating SP population, favored by the higher plating efficiency. All DP clones carried TCR-α/β, since they were $WT31^+$ and $\delta1^-$. Clones isolated from the SP-sorted populations had an SP phenotype. Most of the clones derived from the DN population had the expected $CD4^-8^-$ phenotype and expressed TCR-γ/δ. The rare clones expressing an α/β receptor were CD4 or CD8 SP.

All clones were positive for CD2, CD11a, and CD18 molecules. The range of rela-

TABLE I

*Phenotypic Analysis of Clones Isolated from Thymus and Peripheral Blood*

| Sorted population | Origin | Number of clones positive for:* | | | | | |
|---|---|---|---|---|---|---|---|
| | | CD4-CD8 | CD4 | CD8 | CD3 | WT31 | δ1 |
| $CD4^+8^+$ | Thymus | 81 | 16 | 4 | 101 | 101 | 0 |
| $CD4^+8^-$ | Thymus | 0 | 60 | 0 | 60 | 60 | 0 |
| $CD4^-8^+$ | Thymus | 3‡ | 0 | 65 | 68 | 66 | 2 |
| $CD4^-8^-$ | Thymus | 0 | 3 | 11 | 68 | 7§ | 61 |
| Unsorted | Peripheral blood | 0 | 57 | 32 | 90 | 89 | 1 |

* Clones were obtained from three different thymuses and peripheral blood of the same donors. Cells were stained with OKT3 (anti-CD3), WT31, and δ1 mAbs for single-fluorescence analysis, and with anti-CD4 and anti-CD8 for double-fluorescence analysis.

‡ These three clones expressed low levels of CD4.

§ Three of these were $CD4^+$ and four $CD8^+$.

tive amounts of these molecules, as well as of CD3, CD4, CD8 was comparable in DP and SP clones from both thymus and peripheral blood. All DP clones were also $CD7^+$, Leu $19^+$ and CD1a,b,$c^-$. Remarkably, several DP clones were $CD5^-$ or dull, while the SP clones were always $CD5^+$. Since CD5 is acquired during T cell development (1), and correlates with the acquisition of a mature phenotype (4), the absence of this marker suggests that DP clones represent a population that is not fully differentiated (data not shown).

Thus, the phenotype of DP clones differs from that of DP thymic cells ex vivo. In the thymus, the DP population is heterogeneous, consisting of $TCR^-$, low and high density $TCR^+$, as well as $CD1^+$ and $CD1^-$ cells (13). All clones express high levels of TCR molecules and are $CD1^-$. This might reflect phenotypic changes due to in vitro activation, or a selection exerted by cloning procedures. Therefore, these clones may represent a subset of thymic DP cells.

*Double-Positive Cells Can Switch to Single-Positive In Vitro.* Some DP clones contain a variable percentage (0.5–35%) of SP cells that is relatively stable for each clone over time (Fig. 1). Over a 10-mo period, the bulk of cells remained DP and we never observed a spontaneous shift towards an SP phenotype that involved all the cells. Interestingly enough, some DP clones reproducibly generated $CD4^+$ only, $CD8^+$ only, or both types of SP cells. Similar findings have been reported in short-term cultures of purified DP thymocytes (12), where, however, differential death of DP and overgrowth of contaminating SP cells could not be ruled out. Our experiments clearly demonstrate that this switch most likely occurs at the level of a single clone.

To ask whether this phenomenon is due to a modulation of CD4 or CD8, or to irreversible differentiation, we sorted and expanded from several clones either bright DP cells or SP variants. DP-sorted cells gave rise to the same pattern as the parental clones (see an example in Fig. 2, *a*, *b*). SP variants maintained a stable SP phenotype (see Fig. 2, *c*, *d*), indicating that DP cells can irreversibly differentiate to SP in vitro. It is worth noting that some CD4 SP variants still showed a very low expression

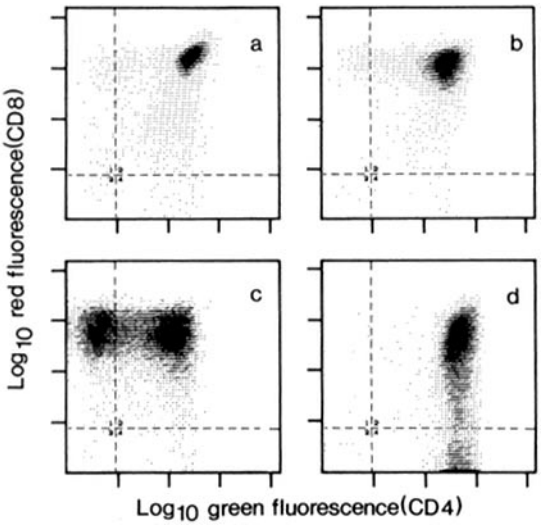


FIGURE 1. Expression of CD4 and CD8 on four representative clones established from DP thymocytes. (*a*, *b*) two DP clones that give rise to very few cells with both CD4 and CD8 SP phenotype. (*c*, *d*) Clones that give rise to more $CD8^+$ or $CD4^+$ variants.

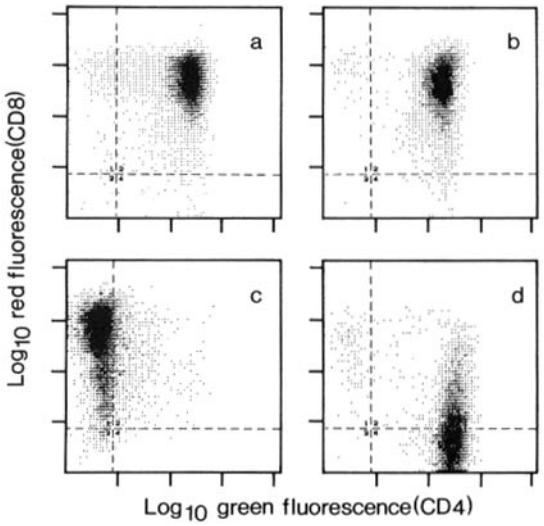


FIGURE 2. CD4 and CD8 expression on a DP clone and on its isolated variants. (*a*) Parental clone; (*b*) cells isolated from the bright DP population; (*c*) cells isolated from single $CD8^+$ variants; (*d*) cells isolated from single $CD4^+$ variants.

of CD8 and we could not isolate variants that were totally $CD8^-$ (see Fig. 2 *d*). However, we could easily isolate $CD8^+$ cells that completely lacked CD4.

The ability to lose independently CD4 or CD8 is peculiar of DP clones and is not observed in peripheral blood- or thymus-derived SP clones.

*Double-Positive Cells Express a Functional TCR-CD3 Complex.* The TCR can transduce a signal in DP clones since these cells are efficiently triggered to cytotoxicity by both anti-CD3 and anti-TCR mAbs (Table II).

The presence of a functional CD3 complex suggests that the TCR can be used by DP cells in their interactions with the thymic microenvironment. These findings are in aggrement with evidence of calcium influx after CD3 triggering in human $CD4^+8^+$ thymocytes (14) and with data identifying the DP subset as target of tolerance induction (8–10). Since thymic tolerance results from deletion of self-reactive cells, it has been suggested that triggering of TCR induces a suicide program in DP thymocytes (15). Our results demonstrate that this is not the general rule for all DP cells, since our clones acquire effector function and proliferate in the presence

TABLE II

*Double-Positive Clones Display a Functional TCR-CD3 Complex*

| | mAbs: | BMA032 | | | OKT3 | | | Medium |
|---|---|---|---|---|---|---|---|---|
| Clone | E/T ratio: | 20:1 | 10:1 | 5:1 | 20:1 | 10:1 | 5:1 | 20:1 |
| Double-positive $\alpha/\beta^+$ | | | | | | | | |
| A31 | | 56* | 49 | 38 | 84 | 71 | 64 | 5 |
| A54 | | 75 | 66 | 49 | 76 | 75 | 67 | 3 |
| A47 | | 38 | 25 | 20 | 62 | 50 | 46 | 4 |
| Double-negative $\gamma/\delta^+$ | | | | | | | | |
| A26 | | 5 | 2 | 4 | 60 | 52 | 43 | 4 |

* Specific $^{51}Cr$ release of P815 target cells obtained in the presence of 1 $\mu$g of BMA032 (anti-TCR-$\alpha/\beta$) and OKT3 (anti-CD3) mAbs.

of anti-CD3 mAbs (data not shown). The possibilities exist that either (*a*) our culture conditions provide an accessory signal that bypasses tolerance induction, or that (*b*) these DP clones represent a subset that is already partially differentiated and therefore no longer tolerizable. More information will be obtained with the study of the TCR repertoire of the DP clones and their SP variants.

## Summary

Human thymocytes were sorted according to the expression of CD4 and CD8 molecules and clones representing the four subpopulations (DP, DN, and single CD4 or CD8 positive) were established. DP clones can be maintained for long periods in tissue culture and give rise to a variable percentage of SP variants. These variants, when isolated and further expanded, do not revert to a DP phenotype. DP clones express a functional TCR-CD3 complex, suggesting that this molecule can interact with the thymic microenvironment during T cell differentiation.

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## References

1. Reinherz, E. L., and S. F. Schlossman. 1980. The differentiation and function of human T lymphocytes. *Cell.* 19:821.
2. Royer, H. D., D. Ramarli, O. Acuto, T. J. Campen, and E. L. Reinherz. 1985. Genes encoding the T-cell receptor $\alpha$ and $\beta$ subunit are transcribed in an ordered manner during intrathymic ontogeny. *Proc. Natl. Acad. Sci. USA.* 82:5510.
3. Collins, M. K. L., G. Tanigawa, A.-M. Kissonergis, M. Ritter, K. S. Price, S. Tonegawa, and J. J. Owen. 1985. Regulation of T-cell receptor gene expression in human T cell development. *Proc. Natl. Acad. Sci. USA.* 82:4503.
4. Lanier, L. L., J. P. Allison, and J. H. Phillips. 1986. Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. *J. Immunol.* 137:2501.
5. Marrack, P., and J. W. Kappler. 1987. The T cell receptor. *Science (Wash. DC).* 238:1073.
6. Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu, and K. Shortman. 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. *Immunol. Rev.* 104:81.
7. Smith, L. 1987. $CD4^+$ murine T cells develop from $CD8^+$ precursors in vivo. *Nature (Lond.).* 326:798.
8. Kisielow, P., H. Blüthmann, U. D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature (Lond.).* 333:742.
9. Fowlkes, B. J., R. H. Schwartz, and D. M. Pardoll. 1988. Deletion of self reactive thymocytes occurs at a $CD4^+8^+$ precursor stage. *Nature (Lond.).* 334:620.
10. Mac Donald, H. R., H. Hengartner, and T. Pedrazzini. 1988. Intrathymic deletion of self-reactive cells prevented by neonatal anti-CD4 antibody treatment. *Nature (Lond.).* 335:174.
11. Havran, W. L., M. Poenie, J. Kimura, R. Tsien, A. Weiss, and J. Allison. 1987. Expres-

sion and function of the CD3-antigen receptor on murine $CD4^+8^+$ thymocytes. *Nature (Lond.).* 300:170.

12. Blue, M.-L., J. F. Daley, H. Levine, and S. F. Schlossman. 1985. Class II major histocompatibility complex molecules regulate the development of the $T4^+T8^-$ inducer phenotype of cultured human thymocytes. *Proc. Natl. Acad. Sci. USA.* 82:8178.
13. Blue, M.-L., J. F. Daley, H. Levine, K. A. Craig, and S. F. Schlossman. 1987. Identification and isolation of a $T4^+T8^+$ cell with high T3 expression in human thymus: a possible late intermediate in thymocyte differentiation. *J. Immunol.* 139:1065.
14. Weiss, A., P. F. Dazin, R. Shields, S. M. Fu, and L. L. Lanier. 1987. Functional competency of T cell antigen receptors in human thymus. *J. Immunol.* 139:3245.
15. Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkinson, and J. J. T. Owen. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature cells in thymic cultures. *Nature (Lond.).* 337:181.