T Cell Receptor–mediated Selection of Functional Rat CD8 T Cells from Defined Immature Thymocyte Precursors in Short-term Suspension Culture

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Summary

Recent results have indicated that positive and negative repertoire selection act on the major population of CD4,8 double-positive (DP) thymocytes that express 5–10-fold less T cell receptor (TCR) than mature T cells (i.e., they are TCR^{low}). Since DP cells obtained ex vivo are heterogeneous with regard to their stage within thymic selection, a homogeneous population of virgin DP cells suitable for selection studies was generated in vitro from their immediate precursors, the CD8 single-positive (SP) immature blast cells. To mimic TCR-mediated selection signals, these virgin DP cells were then cultured for another 2 d in the presence of immobilized anti-TCR monoclonal antibodies with or without interleukin 2 (IL-2). Daily monitoring of recovery and phenotype showed that without TCR stimulation, the cells remained DP and became small, TCR^{low} cells that were lost with a half-life of 1 d, regardless of the presence of IL-2. TCR stimulation resulted in rapid downregulation of CD4 and CD8, maintenance of a larger cell size, and induction of the CD53 antigen that marks mature and CD4,8 double-negative rat thymocytes. In the absence of IL-2, viability decreased as rapidly as without TCR stimulation. Addition of IL-2 rescued TCR-stimulated virgin DP cells and prevented CD8 downregulation, so that 50–80% of input DP cells were recovered after 2 d as $CD4^-8^+53^+$ cells. After release from modulation, these in vitro generated CD8 SP cells quantitatively upregulated the TCR to the TCR^{high} phenotype and were readily induced to proliferate and exhibit cytotoxic T lymphocyte (CTL) activity in a polyclonal readout. Evidence is presented implicating an IL-2 receptor (IL-2R) not containing the p55 chain (i.e., most likely the p70 intermediate affinity IL-2R) in the TCR plus IL-2–driven in vitro differentiation of virgin DP cells towards the mature CD8 SP phenotype.

There is general agreement that intrathymic differentiation of T cells expressing the TCR-α/β proceeds from a TCR-negative, $CD4^-8^-$ (double-negative [DN][1]) via a TCR^{low}, $CD4^+8^+$ (double-positive [DP]) stage towards the mature TCR^{high}, $CD4^+8^-$, and $CD4^-8^+$ (single-positive [SP]) phenotypes. Both positive selection (for self MHC-restriction) and negative selection (for self tolerance) occur during passage through the DP compartment which makes up 90% of immature thymocytes in vivo (For recent reviews, see references 1–3).

To directly demonstrate conversion of one maturational stage to the next, the intrathymic transfer technique has been successfully used with purified DN (4–6) and, more recently, with DP thymocytes (7). In both cases, however, it was found that only subsets of these immature subpopulations give rise to downstream populations (4–7). With regard to DP cells, heterogeneity of precursor cell activity is expected as a result of the ongoing selection process within this compartment. In addition to cells that have already received positive or negative selection signals, unselected cells may be subdivided into those that are still receptive to further differentiation signals (probably the most recently generated DP cells) and those that have lost the potential to respond.

For studies on the differentiation of DP cells in vivo and in vitro, unselected but still selectable cells are obviously the best starting material. For the experiments presently reported, such virgin DP cells were obtained by in vitro maturation of their direct precursors, the immature CD8 SP blasts that represent a brief transitory stage between DN and DP thymocytes (9–12). As first shown by Paterson and Williams (11), such immature CD8 SP cells quantitatively acquire CD4 after overnight culture. Here we report the effects of TCR stimulation on freshly generated DP cells and describe a protocol which for the first time allows the generation of functionally

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; SP, single positive.

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mature CD8 T cells from DP precursors without the need for an intact thymus.

Materials and Methods

Animals. Young adult Lewis rats (Charles River Wiga, Sulzfeld, Germany) of both sexes were used.

Antibodies. mAbs W3/25 and OX-35 (both anti-CD4; references 13 and 14), OX-8 (CD8; reference 13), OX-39 (anti-IL-2R p55; reference 15), OX-44 (reference 16; anti-CD53; reference 16a), R73 (anti-TCR-α/β; reference 17), and 1F4 (anti-CD3; reference 18) were used as dilute ascites for cell separation or were purified and FITC conjugated by standard techniques for use in immunofluorescence. Fab_2 fragments of mAb R73 were prepared and kindly provided by Behringwerke AG (Marburg, Germany). PE-conjugated monoclonal rat anti-mouse-κ antibodies were from Becton Dickinson GmbH (Heidelberg, Germany).

Purification of Cells. Suspensions of thymocytes and LN cells were prepared as described (17). For purification of mature CD8 T cells, LN cells were treated with a saturating concentration of anti-CD4 mAbs and thoroughly washed. 4 μl of a suspension of washed Magnisort-M particles (Dupont Instruments, Wilmington, DE), per 10^6 cells was then added. After 10 min on ice, the mixture was centrifuged for 2 min at 1,500 rpm, gently resuspended, and left for another 10 min on ice. Magnetic particles and adhering cells were then removed in a tissue culture flask fastened to a magnetic plate (Dupont Instruments). This procedure also removes B cells and macrophages, and resulted in >95% pure CD8 cells as assessed by flow cytometry.

Immature CD8 SP thymocytes were prepared by treating thymocyte suspensions with saturating amounts of OX35 and R73 mAbs, and removing the labeled cells by rosetting with rabbit anti-mouse Ig (Dakopatts, Hamburg, Germany)-coated SRBC (19). The remaining cells were sequentially treated with OX44 mAb, biotinylated rabbit anti-mouse Ig (Dakopatts), streptavidin (Sigma Chemical Co., St. Louis, MO), and biotinylated ferritin particles (Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany), before being passed through a magnetic activated cell sorter (MACS; Miltenyi GmbH). For purity see Fig. 1. Viability of freshly isolated and of cultured cells was determined by trypan blue exclusion.

Immunofluorescence and Flow Cytometry. All antibodies were used at saturating concentrations. For two-color immunofluorescence labeling, 5×10^4 to 2×10^5 cells in 100 μl PBS/0.2% BSA/0.02% sodium azide were sequentially exposed for 15 min on ice to (*a*) an unconjugated mAb to the first marker; (*b*) rat anti-mouse-κ PE; (*c*) 10 μg/ml normal mouse IgG; (*d*) FITC-conjugated mAb to the second marker. Specificity of staining and efficacy of blocking were controlled using isotype-matched control mAb. Analysis was performed on a FACScan® flow cytometer (Becton Dickinson GmbH). Light scatter gates were set to include all viable nucleated cells. Samples were analyzed using the FACScan software and are displayed as dotplots or histograms. Populations of marker positive cells were calculated from quadrants set using the appropriate negative controls.

Cell Culture. For in vitro differentiation of thymocytes, 5×10^5 cells/ml were cultured in RPMI 1640 supplemented, as in reference 17, in 24-well plates (NUNC, Kamstrup, Denmark). Where given, the culture wells were precoated overnight with rabbit anti-mouse Ig (40 μg/ml in carbonate buffer pH 9.5) followed by 2 h incubation with mAb R73 at 4 μg/ml in BSS and extensive washing. Human rIL-2 (a kind gift of Hoechst AG, Frankfurt, Germany) was added where given.

Measurement of DNA Synthesis. 10^5 cells were cultured in 0.2 ml of culture medium in 96-well plates (Costar, Cambridge, MA) that, where given, had been indirectly coated with R73 mAb as described above. 0.5 μCi of [^{3}H]thymidine (Amersham Buchler GmbH, Braunschweig, Germany; sp act 2 Ci/mmol) was present during the last 16 h of culture. Incorporation of radioactivity was determined using a Betaplate II liquid scintillation counter (Pharmacia, Freiburg, Germany). Except for very low values, SD were <15%.

Assay for Polyclonal T Cell Mediated Cytotoxicity. 5×10^5 cells/ml were activated in 24-well plates precoated with R73 mAb as described above in the presence of 100 U/m rIL-2. On day 4, cells were recovered, washed, and rested for 4 h at 37°C before adding graded numbers to V-shaped mikrowells containing 10^4 ^{51}Cr-labeled R73 hybridoma or YAC-1 lymphoma cells. Where given, 10-μg/ml Fab_2 fragments of R73 were included in the total volume of 0.2 ml. After 4 h, half of the supernatant was removed and specific lysis was calculated from the released radioactivity according to the formula: percent specific lysis = 100 × (cpm experimental − cpm spontaneous)/(cpm detergent control − cpm spontaneous).

Results

In Vitro Generation of CD4$^+$8$^+$ (DP) Thymocytes. To obtain a population of DP thymocytes free of cells that had already entered TCR-mediated selection or had passed the stage where they can be selected, immature CD8 SP cells were purified and allowed to differentiate to the DP phenotype in overnight suspension culture (11).

Immature CD8 SP thymocytes, which represent ~2% of unseparated thymocytes (16), do not express the OX-44 antigen (the rat homologue of CD53; reference 16a), which is present on mature and on DN thymocytes (16), and are TCR negative to very low (17). Accordingly, thymocytes from young adult rats were depleted by indirect rosetting and magnetic cell sorting using anti-CD53, anti-CD4, and anti-TCR mAbs. Routinely, 5×10^6 purified immature CD8 SP cells were obtained in this fashion from one thymus (10^9 thymocytes). Their phenotype is shown in Fig. 1. As previously noted (17, 20) immature CD8 SP cells are large blasts that express very low levels of the CD3/TCR complex (visualized in Fig. 1 with an anti-CD3 mAb since anti-TCR had been used for separation), and no detectable IL-2R p55 chain (CD25). In accordance with their cycling status (11), immature CD8 SP cells incorporated [^{3}H]thymidine (Fig. 2) at the beginning of cell culture, but in agreement with observations by others (11), had stopped cycling by day 1.

The properties of the virgin DP cells collected after overnight cell culture, which were the starting population for all further experiments, are also illustrated in Fig. 1. >95% now expressed both CD4 and CD8. The cell size had decreased but was still above that of the small DP cells that make up the majority of immature thymocytes in vivo. As previously reported (20) some of the in vitro generated DP cells had already upregulated the CD3/TCR complex to a level characteristic of their in vivo counterparts (TCR^{low}), which is about 10-fold less than that expressed by the minor population of mature thymocytes (TCR^{high}) contained within unseparated thymocytes (Fig. 1, *top*).

The recovery of DP thymocytes after overnight culture was

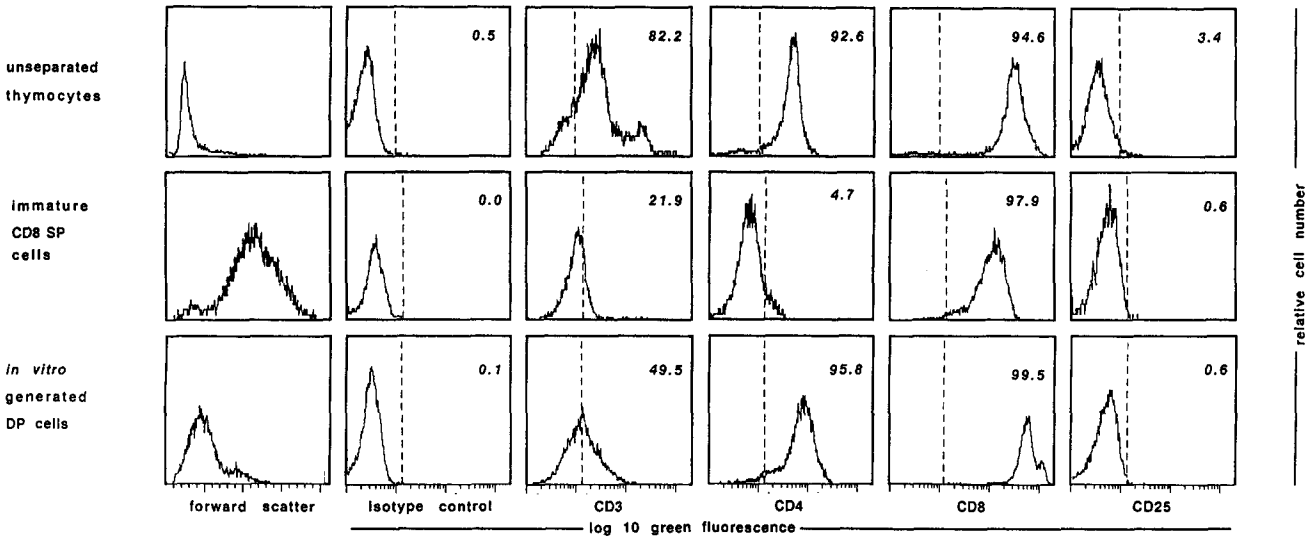

Figure 1. Phenotype of in vitro generated DP thymocytes and their CD8 SP immediate precursors. Unseparated and immature CD8 SP thymocytes were analyzed by immunofluorescence and flow cytometry immediately after isolation. In vitro generated DP cells were immature CD8 SP cells cultured for 24 h in medium.

100–120% of immature CD8 SP input cells. The further in vitro differentiation potential of these freshly generated DP cells was next investigated.

In Vitro Differentiation of Newly Formed DP Thymocytes: Experimental Design. Since by definition, positive and negative selection of thymocytes involves ligation of the TCR, newly generated DP thymocytes were cultured in dishes precoated with anti-TCR mAb. IL-2 was added to one set of cultures and [^{3}H]thymidine incorporation (Fig. 2), viability (Fig. 3), and cell surface phenotype (Fig. 4) were monitored during the following 3 d (i.e., days 2–4 of culture), and compared to cultures where no TCR crosslinking was performed.

To illustrate the dynamics of the subpopulations visualized in Fig. 4, the number of cells shown in each dot plot is proportional to the number of viable cells recovered in that group. In addition, the representation of the various phenotypes is calculated and summarized in Table 1. These data are representative of more than 10 individual experiments which yielded virtually identical results.

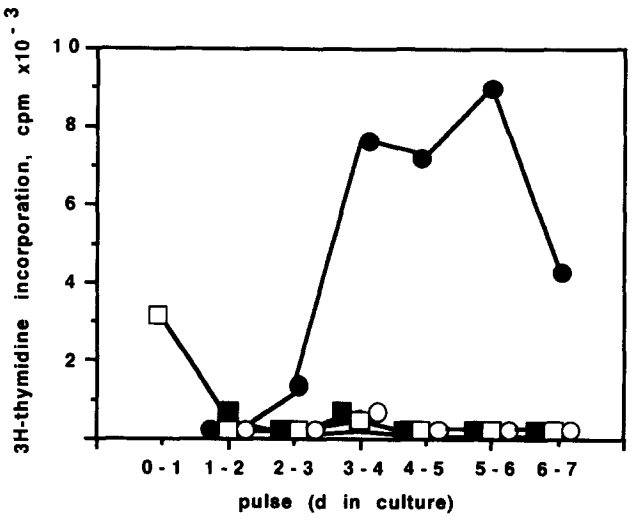

Figure 2. [^{3}H]thymidine incorporation in cultures of immature CD8 SP thymocytes. From day 0 to day 1, all cells were cultured in medium only. For the remaining culture period, they were kept in untreated (*squares*) or anti-TCR mAb-coated wells (*circles*), in the absence (*open symbols*) or presence (*filled symbols*) of 100 U/ml rIL-2, and pulsed for 16 h between the days indicated.

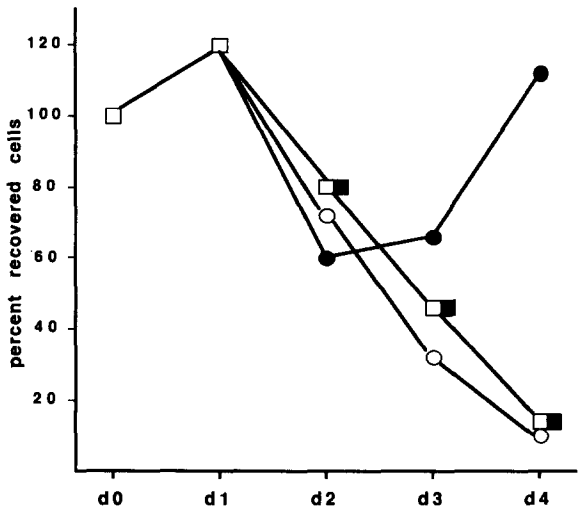

Figure 3. Cell recovery upon cultivation of immature CD8 SP thymocytes. Immature CD8 SP thymocytes were cultured in medium for 1 d. For the following 3 d, they were kept in untreated (*squares*) or anti-TCR mAb-coated wells (*circles*), in the absence (*open symbols*) or presence (*filled symbols*) of 100 U/ml rIL-2. Recovery of viable cells was determined by trypan blue exclusion.

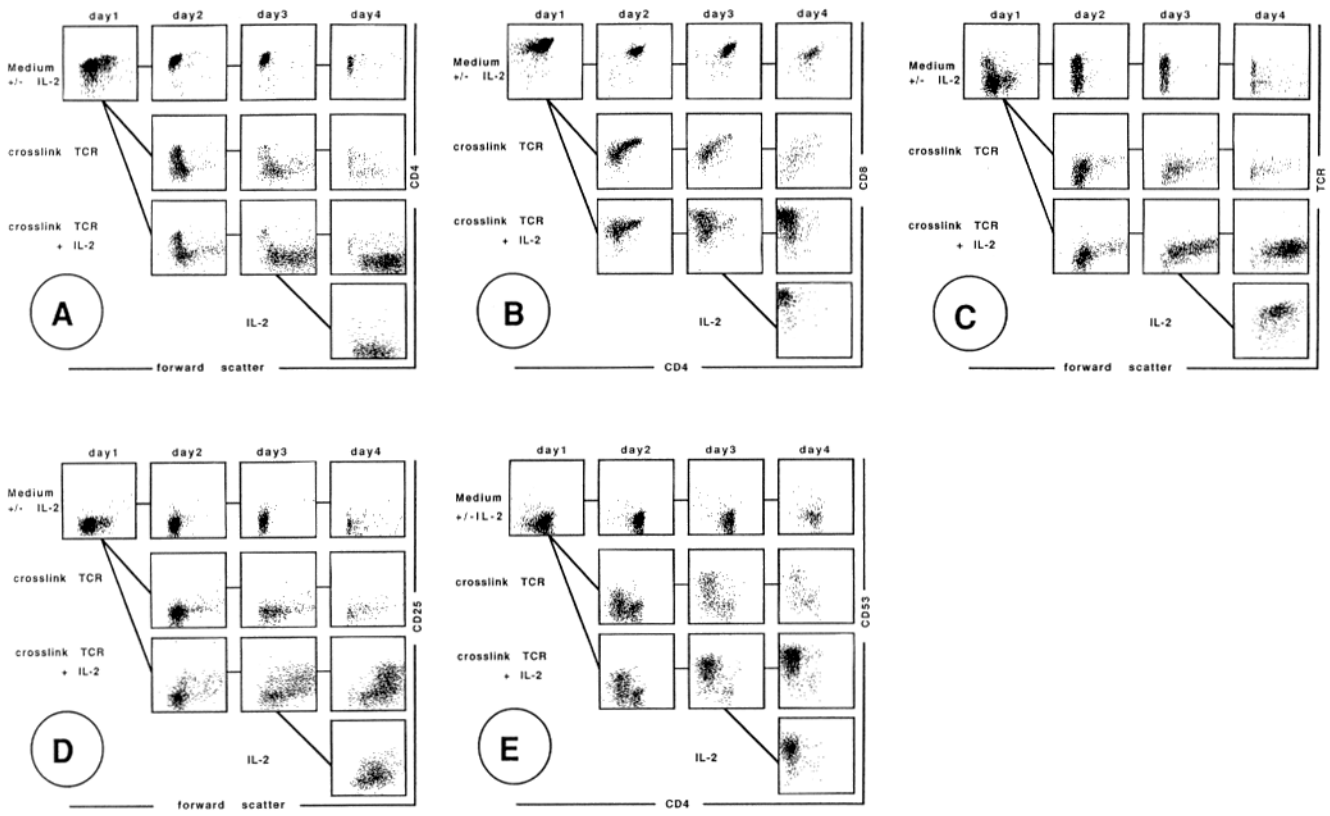

Figure 4. Phenotype of in vitro generated DP cells upon further in vitro cultivation. 5×10^5 DP cells/ml collected on day 1 from cultures of CD8 SP precursors were cultured under the conditions given. Samples were analyzed daily by two-color flow cytometry. The number of cells shown is proportional to the cell recovery (2000 cells being shown for the starting population, day 1). For recoveries see Fig. 3 and for representation of phenotypes see Table 1.

Maintenance of TCR^{low} DP Phenotype in Absence of Receptor Stimulus. Upon further cultivation of in vitro generated DP cells in medium only, no DNA synthesis was observed (Fig. 2). Rather, the number of viable cells was halved every day (Fig. 3). At the same time the remaining cells became smaller (Fig. 4 *A*), approaching forward light scatter values characteristic of small cortical DP thymocytes. Throughout the culture period, the cells maintained their CD4,8 DP phenotype (Fig. 4 *B*), and the fraction of TCR^{low} cells increased to ~70% (Fig. 4 *C*, Table 1). In contrast, CD25 and CD53 remained undetectable (Fig. 4 *D* and *E*). All of these phenotypic properties (and the expression of a number of additional cell surface markers, not shown) make DP cells that have been generated and further cultured in vitro, indistinguishable from the bulk of DP thymocytes found in vivo.

In the presence of rIL-2 (up to 500 U/ml), newly generated DP thymocytes behaved exactly as those cultured in medium alone. Since cultures with or without IL-2 were indistinguishable in every aspect studied, only one set of phenotypic analyses is shown in Fig. 4 for both groups.

Effects of TCR Ligation on Newly Generated DP Thymocytes. If the TCR was crosslinked in the absence of IL-2, viable cells were lost at the same rate as when cultured in medium only (Fig. 3), and again, no thymidine incorporation was detectable throughout the culture period (Fig. 2). Flow cytometric analysis revealed, however, that TCR crosslinking had not been without effect: a fraction of cells corresponding in number to the TCR^{low} population in cultures without anti-TCR mAb, had down-regulated both CD4 and CD8, the effect being more pronounced on CD4 than on CD8 expression (Fig. 4 *B*). In addition, the cells that had responded to TCR stimulation with CD4 downregulation, remained larger than unstimulated controls (Fig. 4 *A*) and began to express the CD53 antigen (Fig. 4 *E*). No CD25$^+$ cells were observed throughout the culture period (Fig. 4 *D*), and as expected, the TCR was modulated (Fig. 4 *C*). In summary, TCR crosslinking led to downregulation of CD4 and CD8, expression of CD53, and maintenance of a larger cell size, but did not induce CD25 or prevent cell death.

Addition of IL-2 Rescues TCR-stimulated DP Cells. To our surprise, addition of rIL-2 to TCR-stimulated DP cells had a dramatic influence on cell recovery despite the initial absence of the IL-2R p55 chain. Thus, when virgin DP cells were cultured in the presence of both crosslinked anti-TCR mAb and IL-2, the cell number stabilized after 1 d and by day 3 of stimulation (i.e., day 4 of culture) increased to input

Table 1. *Phenotype Composition of Cell Cultures Derived from Immature CD8 SP thymocytes*

d in culture	Culture conditions	$CD4^-8^+$	$CD4^+8^-$	$CD4^+8^+$	TCR^+	$CD53^+$	$CD25^+$
0		98.4	<1	<1	ND	<1	<1
1	Medium	2.6	<1	95.4	14.8^{low*}	<1	<1
2	Medium	<1	<1	98.8	56.8^{low}	<1	2.0
2	IL-2	<1	<1	98.5	54.9^{low}	<1	<1
2	Crosslinked TCR	38.5	<1	58.9	mod	9.2	2.8
2	Crosslinked TCR/IL-2	41.7	<1	53.8	mod	42.8	18.5
3	Medium	<1	<1	98.9	73.3^{low}	5.1	4.5
3	IL-2	<1	<1	98.4	69.8^{low}	3.8	2.9
3	Crosslinked TCR	57.1	<1	30.6	mod	55.8	1.0
3	Crosslinked TCR/IL-2	78.2	<1	14.4	mod	79.5	32.6
4‡	Medium	4.2	<1	91.8	44.7^{low}	16.9	1.3
4‡	IL-2	3.8	<1	93.7	49.9^{low}	12.1	2.2
4‡	Crosslinked TCR	33.5	<1	34.2	mod	54.2	3.9
4	Crosslinked TCR/IL-2	92.2	<1	1.6	mod	90.4	48.8
4	Crosslinked TCR/IL-2 d 1+2, IL-2 only d 3	97.9	<1	1.5	92.5^{high}	94.9	27.1

5×10^5 immature CD8 SP cells/ml were cultured for 1 d in medium, readjusted to 5×10^5, and cultured for another 3 d under the conditions given. Samples were analyzed daily by two-color flow cytometry and populations calculated by quadrant analysis using negative controls. Data are given as percent of viable recovered cells. mod, modulated.

* low, high: levels of TCR expression characteristic of immature vs. mature thymocytes.

‡ Result likely to be influenced by low cell recovery (<10% of input, see Fig. 3).

values (Fig. 3). As expected, the doubling in cell number at this final stage of cell culture was reflected in terms of thymidine incorporation which was upheld for at least another 3 d (Fig. 2). In contrast, no DNA synthesis was observed during the first, and only very little during the second day of stimulation with anti-TCR mAb plus IL-2. During this initial phase of stimulation without DNA systhesis, the phenotype of the DP input cells changed dramatically (Fig. 4, Table 1): CD4, but not CD8 was lost from the cell surface, CD53 was strongly induced, and the p55 chain of the IL-2R became detectable. It is worth noting, however, that expression of CD25 lagged behind CD4 down- and CD53 upregulation and was detected on only about half of the cells recovered at any of the time points studied.

In summary, after 2 d of stimulation of virgin DP cells by anti-TCR mAb plus IL-2 (i.e., on day three of cell culture), more than half of input cells (i.e., most of the cells that were potential targets for TCR stimulation) were recovered. They were of the $CD4^-8^+53^+$ and, in part, $CD25^+$ phenotype (Fig. 4, Table 1). While this differentiation occurred in the absence of DNA synthesis, continued stimulation by anti-TCR mAb plus IL-2 initiated clonal expansion.

Rescue of DP Thymocytes by Anti-TCR mAb Plus IL-2 Requires High Lymphokine Concentrations. In the above experiments, IL-2 was used at a concentration of 100 U/ml. From the results shown in Fig. 5 *A*, it is apparent that low doses of IL-2 that are sufficient to induce proliferation in TCR-stimulated peripheral T cells (see below) have little effect in rescuing TCR-stimulated DP thymocytes. In keeping with the requirements for and kinetics of CD25 expression described above, this suggests that at least initially, IL-2 acts on TCR-stimulated DP thymocytes via intermediate-affinity (p70) receptors.

DP Thymocytes Rescued by Anti-TCR mAb Plus IL-2 Are Phenotypically Mature CD8 SP T Cells. Newly generated DP thymocytes were stimulated for 2 d with anti-TCR2 mAb plus IL-2. To investigate the level of TCR expression on these in vitro differentiated $CD4^-8^+53^+$ cells, they were cultured for an additional day (i.e., from day 3 to day 4 of culture) without mAb to release the TCR from modulation. The results are included in Fig. 4, and a direct phenotypic comparison with peripheral CD8 T cells that were either freshly isolated or put through exactly the same cell culture protocol as used for the differentiation of immature CD8 SP thymocytes is shown in Fig. 6. All recovered cells (50–70% of immature CD8 SP input cells) now expressed as high levels of the TCR as did mature T cells. In addition, they quantitatively maintained the $CD4^-8^+53^+$ phenotype. They were, however, at least in part, larger than freshly isolated mature

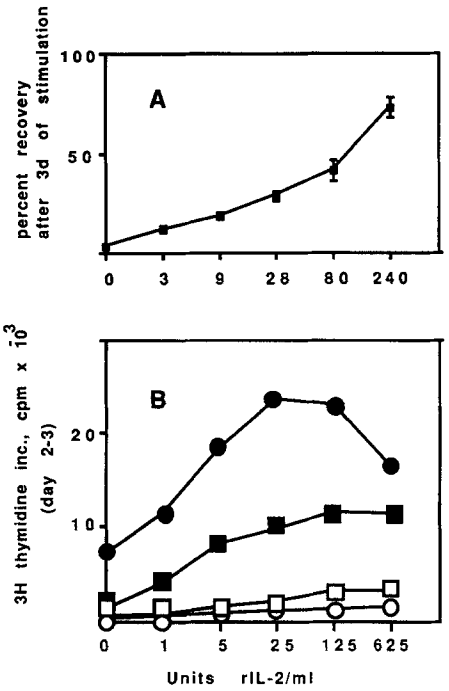

Figure 5. IL-2 dependence of in vitro differentiation of virgin DP thymocytes and of proliferation induced in their mature progeny. (*A*) 2 × 10^5 newly generated DP thymocytes were cultured for 3 d in .4 ml medium in the presence of crosslinked anti-TCR mAb and increasing concentrations of IL-2. (*B*) In vitro differentiated CD8 SP cells (*squares*) or freshly isolated peripheral CD8 T cells (*circles*) were cultured in the presence (*filled symbols*) or absence (*open symbols*) of crosslinked anti-TCR mAb and increasing concentrations of IL-2. Cultures were pulsed with [^{3}H]thymidine for 16 h from day 2 to 3 of culture.

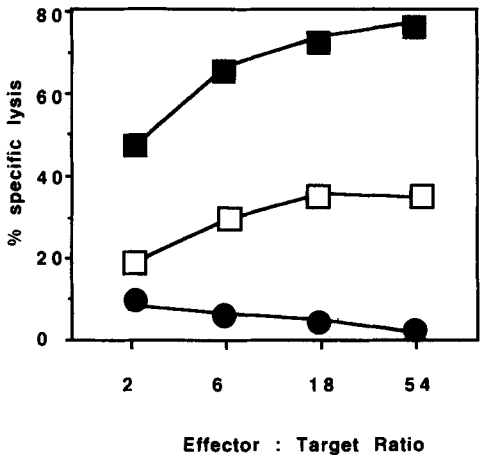

Figure 7. Cytolytic activity of in vitro differentiated and activated CD8 SP T cells. Immature CD8 SP thymocytes were allowed to mature in vitro as described in the text. After 4 d of activation with crosslinked anti-TCR mAb, cytolytic activity was determined in a standard 4 h ^{51}Cr release assay against YAC-1 targets (*circles*), or against R73 hybridoma cells in the absence (*open squares*) or presence (*filled squares*) of 10 μg/ml of purified R73 Fab_2 fragment.

CD8 T cells, and a minority expressed low levels of CD25. In contrast, mature CD8 T cells cultured under identical conditions were, without exception, blast cells and mostly CD25$^+$. Taken together, these results suggested under our cell culture conditions, TCR^{high} CD8 SP cells had differentiation from virgin DP precursor cells.

Functional Capacity of In Vitro Generated CD8 SP T Cells. Using the protocol developed in the experiments described above, sufficient numbers of CD8$^+$TCR^{high} cells were generated in vitro to perform functional studies. Routinely, 1–2 × 10^7 highly purified immature CD8 SP cells were cultured overnight in medium, then stimulated for 2 d with crosslinked anti-TCR mAb plus 100 U/ml of IL-2, and rested for 1 d in medium containing IL-2 only. The purity was moni-

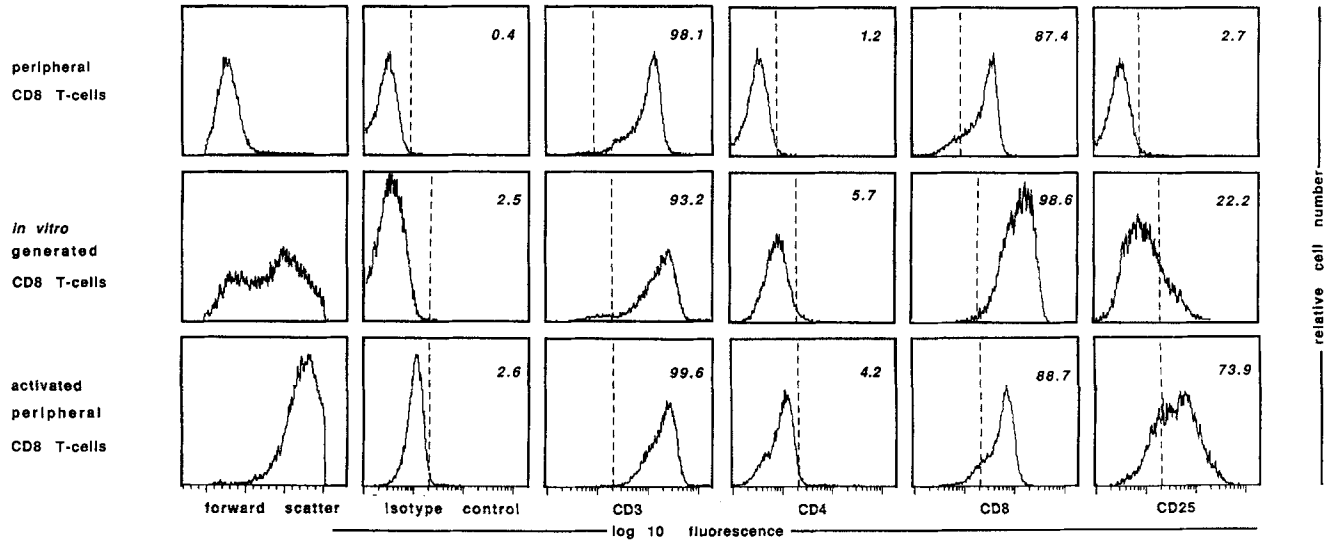

Figure 6. Phenotypic comparison of in vivo and in vitro differentiated CD8 T cells. Freshly isolated peripheral and in vitro differentiated CD8 T cells were analyzed by immunofluorescence and flow cytometry. For comparison, peripheral CD8 T cells that underwent the in vitro differentiation protocol used for CD8 immature thymocytes are shown (activated peripheral T cells).

tored on days 0, 1, and 4 of culture and was always >95% with respect to the expected stage of differentiation. Recovery was between 50–80% of input cells. In the experiment shown in Fig. 5 *B*, the proliferative responses of in vitro generated and of freshly isolated CD8 SP T cells were compared. Both populations incorporated [^{3}H]thymidine when stimulated with anti-TCR mAb plus recombinant IL-2. DNA-synthesis of freshly isolated cells was, however, two-fold higher than that of in vitro generated CD8 T cells. Note that much lower amounts of IL-2 were required to induce proliferation in both populations than were needed for differentiation and rescue of TCR-stimulated DP cells.

TCR-mediated CTL Activity of In Vitro Differentiated and Restimulated CD8 T Cells. CD8 T cells generated in vitro as described above were polyclonally activated using immobilized anti-TCR mAb and IL-2. On day 4, CTL activity was measured using the anti-TCR hybridoma R73 as target. In addition, cytotoxic activity against the (mouse and rat) NK-sensitive mouse YAC-1 lymphoma was studied. As shown in Fig. 7, R73, but not YAC-1 targets were efficiently lysed. Moreover, Fab_2 R73 mAbs inhibited lysis of the R73 hybridoma. Thus, TCR-mediated CTL activity, but not NK-cell activity, is readily detected in in vitro generated CD8 SP T cells after activation with anti-TCR mAb and IL-2.

Discussion

The present paper demonstrates for the first time that phenotypically and functionally mature CD8 T cells can be efficiently generated in short-term suspension culture from immature CD8 SP precursors via a DP intermediate. Crucial to the interpretation of these results is the certainty that these cells were not derived from contaminating mature thymocytes. The following points make this possibility highly unlikely: (*a*) The virgin DP cells collected after overnight differentiation of CD8 immature thymocytes contained no detectable (i.e., far <1%) TCR^{high} or $CD53^+$ cells. Since the number of TCR^{high}, $CD53^+$CD8 SP T cells collected only 3 d later amounted to 50–70% of input cells, it is very unlikely that they were the progeny of a contamination too small to detect by flow cytometry. (*b*) Downregulation of CD4 and expression of CD53 occurred within a day on most, if not on all, of the cells that were potential targets for stimulation by anti-TCR mAb, i.e., that expressed the TCR (Table 1, Fig. 4). The same holds true for the stabilization of a $CD8^{high}$ phenotype by IL-2 on TCR-stimulated DP cells (Fig. 4). Thus, the phenotypic properties that characterized the end product were rapidly induced on the majority of cells in the absence of detectable DNA synthesis. (*c*) Whereas all activated peripheral rat T cells express the p55 chain of the IL-2R (15), CD25 was expressed on less than half of the cells that were rescued from the DP population as a consequence of stimulation by anti-TCR mAb plus IL-2 (Table 1). Accordingly, IL-2 must have acted through a receptor lacking the p55 chain, presumably through the intermediate affinity p70 IL-2R (21). Since neither addition of IL-2 nor TCR crosslinking by themselves resulted in CD25 expression, it seems likely that TCR-crosslinking induced only the IL-2R p70 chain, a situation not observed in mature rat T cells which quantitatively become $CD25^+$ after TCR-stimulation (15, 17). Those differentiation products of DP cells that did express CD25 as a result of anti-TCR plus IL-2 stimulation are likely to have progressed to maturity and gone on to clonally expand (note their blastoid phenotype, Fig. 4 *D*). In fact, as can be seen in Fig. 2, DNA-synthesis which became detectable between day 2 and 3 of culture steeply increased during the next day and was upheld for at least three more days in the presence of anti-TCR mAb plus IL-2, suggesting that a resting phase between differentiation and clonal expansion is not required. Indeed, functional cytotoxic cells were also induced by nonstop differentiation plus activation (not shown).

Why were only CD8 T cells generated? Since mature CD4 SP cells arise upon intrathymic transfer of mouse DP blasts (7), DP cells must also contain the precursors for CD4 T cells. One possibility is that the immature CD8 SP starting population is already committed to the CD8 lineage and that, accordingly, the virgin DP cells studied were not representative of virgin DP cells in vivo. We consider this unlikely because at least in the mouse, intrathymic transfer of immature CD8 SP cells gives rise to both CD4 and CD8 SP mature subpopulations (7). Furthermore, when DP cells isolated ex vivo were used for in vitro differentiation experiments, stimulation by anti-TCR mAb plus IL-2 again led to downregulation of CD4, maintenance of CD8, and expression of CD53, although only fewer of these cells could be driven to maturity (not shown). Accordingly, it must be the quality of the signals provided in our system and not that of the DP precursor cells selected that is responsible for the sole generation of CD8 T cells. It is worth noting that unfractionated mouse DP thymocytes downregulate CD4 when stimulated with PMA (22), suggesting that this mechanism is operative in all DP cells, regardless of their stage of progression within the DP compartment.

Our results demonstrate that in vitro, TCR engagement plus high doses of IL-2 drive freshly generated DP cells towards the CD8 SP phenotype and to maturity. This does not necessarily mean that this pathway is operative in vivo. It is equally possible that the IL-2R is functional but not used during positive selection in vivo, so that the effect of IL-2 presently described bypasses an unknown physiological stimulus. In addition, the signals required for selection of CD4 SP from DP cells in vitro or in vivo remain completely unclear. Following a suggestion by Emmrich et al. (23), we have stimulated DP cells with coimmobilized anti-TCR and anti-CD4 mAbs (plus IL-2), but again obtained only CD8 SP cells. A number of other protocols (different lymphokines, epithelial cells, etc.), however, remain to be tested. It may be of interest that anti-TCR stimulation by itself leads to downregulation of both CD4 and CD8, and that IL-2 selectively prevents CD8 downregulation (Fig. 4 *B*). Thus, the hypothetical signal that leads to the generation of $CD4^+8^-$ cells may be one that selectively prevents disappearance of CD4 from the cell surface.

The generation of mature CD8 SP T cells by the present straightforward protocol give assess to an unselected TCR repertoire expressed on functional cells. Unfortunately, anal-

ysis is hampered by the lack of mAbs defining anti-TCR Vβ segments in the rat as have been described for the mouse. Conversely, it is extremely difficult to purify sufficient numbers of mouse immature CD8 cells to perform such repertoire studies in that species. Thus, evaluation of allo- an selfreactivity are the only tools presently available to look at the rat TCR repertoire. This approach is currently being pursued to compare the CD8 T cell repertoire generated inside versus outside an intact thymus.

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