Brief Definitive Report

T Cell Receptor Ligation Induces Interleukin (IL) 2R β Chain Expression in Rat CD4,8 Double Positive Thymocytes, Initiating an IL-2–dependent Differentiation Pathway of CD8α⁺/β⁻ T Cells

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Summary

The role of interleukin (IL)2 in intrathymic T cell development is highly controversial, and nothing is known about IL-2R expression on thymocytes of the T cell receptor (TCR) α/β lineage undergoing TCR-driven differentiation events. We analyze here IL-2R α and β mRNA expression in an in vitro system where newly generated rat CD4,8 double positive (DP) thymocytes respond to TCR ligation plus IL-2 (but not to either stimulus alone) with rapid differentiation to functional CD8 single positive T cells (Hünig, T., and R. Mitnacht. 1991. *J. Exp. Med.* 173:561). TCR ligation induced expression of IL-2R β (but not α) chain mRNA in DP thymocytes. Addition of IL-2 then lead to functional maturation and expression of the IL-2R α chain. To investigate if the CD8 T cells generated via this IL-2R β-driven pathway in vitro correspond to the bulk of CD8 T cells seeding peripheral lymphoid organs in vivo, we compared their phenotype to that of lymph node CD8 T cells. Surprisingly, analysis of CD8 cell surface expression using a novel anti-CD8 monoclonal antibody specific for the α/β heterodimeric isoform, and of CD8 α and β chain mRNA revealed that T cells generated by TCR ligation plus IL-2 resemble thymus-independent rather than thymus-derived CD8 cells in that they express CD8 α without β chains. These findings demonstrate that TCR crosslinking induces functional IL-2R on immature DP rat thymocytes. In addition, they show that at least in vitro, CD8α/α T cells are generated from TCR-stimulated DP thymocytes (which express the CD8α/β in the heterodimeric isoform) along an IL-2–driven pathway of T cell differentiation.

The receptors and signals that guide immature thymocytes of the α/β TCR lineage towards the mature CD4 and CD8 single positive (SP) phenotypes are incompletely understood. In addition to the antigen receptor itself and its coreceptors CD4 and CD8, cytokines and cell interaction molecules are thought to be involved in regulating expansion and differentiation of immature precursor cells. With regard to the lymphokine IL-2, a large and in part contradictory body of experimental evidence exists favoring (1–3) or discarding (4, 5) its central role in thymopoiesis (for review see reference 6). Moreover, recent studies on genetically IL-2–deficient mice have ruled out an essential function of IL-2 in mainstream T cell development, at least in this species (7). The significance of IL-2 (8, 9) and of IL-2R expression in TCR-negative immature mouse thymocytes (3, 10, 11) thus remains unclear. Whereas the function of IL-2R in the TCR negative preselection compartment is uncertain, no information exists about IL-2R on cells of the TCR^{low} DP compartment subject to TCR-mediated selection events.

A striking effect of IL-2 on thymocyte differentiation within this compartment has been observed in rats, where newly generated ("virgin") double positive (DP) thymocytes rapidly convert in vitro to mature CD8 SP T cells in response to immobilized TCR-specific mAb plus IL-2, but not in response to either stimulus alone (12). Here we analyze the expression of IL-2R chains in this in vitro differentiation system and ask whether the CD8 T cells generated in vitro by TCR ligation plus IL-2 correspond to those generated in mainstream T cell development in vivo.

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; 13, 14), OX-8 (anti-CD8; 13), OX-44 (15; anti-CD53; 16), R73 (anti-TCR-α/β; 17), 1F4 (anti-CD3; 18), and 341 (anti-CD8, reactive only with molecules containing the β chain; 19) were used as dilute ascites or tissue culture supernatant. For use in immunofluorescence, mAbs were purified and FITC conjugated by standard tech-

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niques. PE-conjugated F(ab')$_2$ donkey anti-mouse was from Dianova GmbH (Hamburg, Germany).

Immunofluorescence and Flow Cytometry. All antibodies were used at saturating concentrations. For two-color immunofluorescence labeling (12), 5×10^4–2×10^5 cells in 100 μl PBS/0.2% BSA/0.02% sodium azide were incubated for 15 min on ice with an unlabeled mAb, followed by 15 min treatment with F(ab')$_2$ donkey anti-mouse-PE, 10 μg/ml normal mouse IgG, and FITC-conjugated mAb to the second marker. Specificity of staining was confirmed using isotype-matched control mAb. Analysis was performed on a FACScan® flow cytometer (Becton Dickinson GmbH, Mountain View, CA). 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.

Preparation of Cells. Suspensions of thymocytes were prepared as described (12). Immature CD8 SP thymocytes were isolated by treating thymocyte suspensions with a saturating amount of R73 and W3/25 mAbs and removing the labeled cells by rosetting with rabbit anti-mouse Ig (Dakopatts, Hamburg, Germany) -coated SRBC (20). The remaining cells were sequentially treated with OX35 and 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). The resulting population consisted of more than 97% immature CD8 SP cells with a viability >90% as determined by trypan blue exclusion.

Cell Culture (12). 5×10^5 thymocytes/ml RPMI 1640 supplemented as in reference 17 were cultured in 12-well plates (NUNC, Kamstrup, Denmark). For TCR stimulation, 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. 500 U/ml human rIL-2 (a kind gift of Hoechst AG, Frankfurt, Germany) was added where given.

Preparation of Cytoplasmic RNA. Cytoplasmic RNA was isolated by ethanol precipitation from cytoplasmic NP-40 extracts following the method described by Gough (21) as modified by Schreiber et al. (22).

RNase Protection Assays. RNase protection assays were performed according to the method of Melton et al. (23). cDNA encoding rat IL-2R α and β chains (24) were the kind gift of Dr. M. Dallman (Oxford, UK). cDNA encoding rat CD8α (25) and β (26) were the kind gift of Dr. A. Williams (Oxford, UK). For the IL-2R p55 chain, a ^{32}P-labeled 390-bp-long negative strand RNA (protected fragment size: 360 bp) was transcribed from a PstI/PvuII cDNA fragment subcloned into pGEM-2 (Promega Corp., Madison, WI). For the p70 chain, an 870-bp-long negative strand RNA (protected fragment size: 827 bp) was transcribed from a pBluescriptSK (Stratagene Corp., La Jolla, CA) vector containing the cDNA upstream of the first BglII site. CD8 mRNAs were transcribed from pGEM-3 vectors (Promega Corp.) containing either the 600-bp BamHI/EcoRI fragment of rat CD8α cDNA or the 614-bp NcoI/PstI fragment of CD8β cDNA, resulting in protected fragments of 360 and 617 bp, respectively. The β-actin transcription vector pSPβact72 constructed by M. Jantzen containing a 280-bp fragment (protected fragment size: 280 bp) of the rat β-actin cDNA (27) was kindly provided by F. Siebelt.

Results

TCR Stimulation Primes DP Thymocytes for IL-2–driven Differentiation to CD8 SP Cells. Highly purified rat CD4,8 DP thymocytes without prior in vivo exposure to selecting signals were obtained by isolating their direct precursors, the immature CD8 SP blast cells, and allowing them to differentiate overnight in cell culture (12, 28). We have previously described in detail (12) that: (*a*) such virgin DP cells during further incubation in medium with or without exogenous IL-2 acquire the phenotype characteristic of cortical thymocytes, i.e., they become small DP cells with a TCR level 5–10 times below that of mature T cells; (*b*) stimulation of virgin DP thymocytes with immobilized anti-TCR mAb leads to down-regulation of both CD4 and CD8; and (*c*) IL-2 rescues about half of TCR-stimulated virgin DP thymocytes from cell death and induces their differentiation to functionally mature CD8 SP cells within 2 d. Fig. 1 illustrates the changes in CD4 and CD8 expression in this in vitro differentiation system. Note that not all virgin DP cells respond to the immobilized TCR-specific mAb, since as DP cells isolated ex vivo, they contain a TCR-negative subpopulation (12).

The requirement for both anti-TCR mAb and IL-2 for differentiation of virgin DP to CD8 SP cells could either mean that virgin DP cells already carried IL-2R, but that both the TCR- and the IL-2R–mediated signals were required simultaneously, or that TCR stimulation induced IL-2 reactivity, most likely by the induction of IL-2R. To distinguish between these possibilities, virgin DP cells were cultured for 1 d in the presence of immobilized TCR-specific antibodies, followed by culture in IL-2. As is shown in the lower half of Fig. 1, virgin DP cells cultured for 1 d in dishes coated

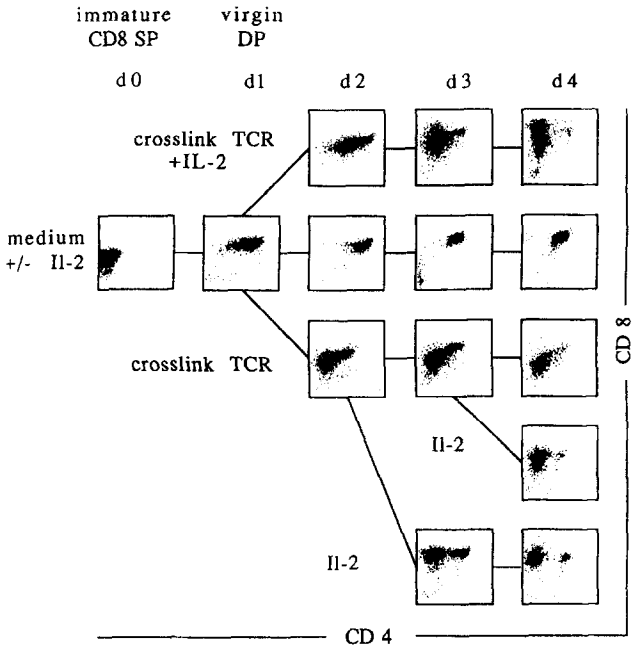

Figure 1. TCR stimulation primes DP thymocytes for IL-2–driven differentiation. Virgin DP thymocytes harvested from overnight cultures of immature CD8 SP thymocytes (*immature CD8 SP*) were cultured for the times indicated in untreated wells or in wells coated with TCR-specific mAb R73 (*crosslink TCR*), in the presence or absence of 500 U/ml IL-2. CD4/8 phenotype of 5,000 cells as determined by two-color immunofluorescence and flow cytometry is shown.

with TCR-specific mAb differentiate to CD8 SP cells upon subsequent culture in the presence of IL-2, whereas continuous culture in IL-2 without TCR stimulation is without effect. We next investigated this priming effect at the level of IL-2R expression.

Induction of IL-2R mRNA in Virgin DP Thymocytes. Two types of functional IL-2 receptors have been described: high affinity receptors, composed of at least the α (55 kD) and the β (70 kD) chain, and intermediate affinity receptors lacking the α chain (29). When ex vivo isolated immature CD8 SP thymocytes and their 1-, 2-, or 3-d-old DP progeny were investigated for the presence of IL-2R α and β chain mRNA by RNase protection analysis, no IL-2R transcripts were observed (Fig. 2). Addition of 500 U/ml IL-2 also failed to induce IL-2R mRNA, a result that is in keeping with the complete lack of an effect of IL-2 on phenotype or survival of unstimulated virgin DP cells (12 and Fig. 1). It is interesting, however, that TCR crosslinking by an immobilized mAb induced IL-2R β but not α chain mRNA during overnight culture. The signal observed was comparable in strength to that found in Con A-activated spleen cells and remained constant when cells stimulated for 48 h were examined, providing an explanation for the effects of IL-2 added 1 or 2 d after TCR stimulation (Fig. 1).

When TCR-stimulated cultures were supplemented with IL-2, there was an additional expression of IL-2R α chain and a further increase in the level of β chain mRNA. These results are in agreement with the dependence of CD25 cell surface expression in TCR-stimulated virgin DP cells on exogenous IL-2 (12). Since the IL-2R β chain is essential for signaling by IL-2 (30), these data furthermore indicate that IL-2 induces the α chain of its receptor via IL-2R containing β but not α chains.

Induction of IL-2Rβ mRNA by TCR stimulation and the subsequent transcription of both IL-2R chains upon further addition of IL-2 was not restricted to virgin DP cells generated in vitro, but was also observed with ex vivo isolated DP thymocytes (data not shown).

IL-2 Selectively Rescues the CD8 α chain on TCR-stimulated DP Cells. The recent finding that in IL-2–deficient mice, intrathymic T cell differentiation is apparently unaffected (7), prompted us to reexamine whether the functionally mature CD8 T cells, generated in vitro by stimulation of virgin DP thymocytes with TCR-specific mAbs and IL-2, were the counterparts of CD8 T cells derived from mainstream intrathymic maturation in vivo. By phenotypic analysis employing a panel of mAbs to cell surface markers including CD3, CD2, and

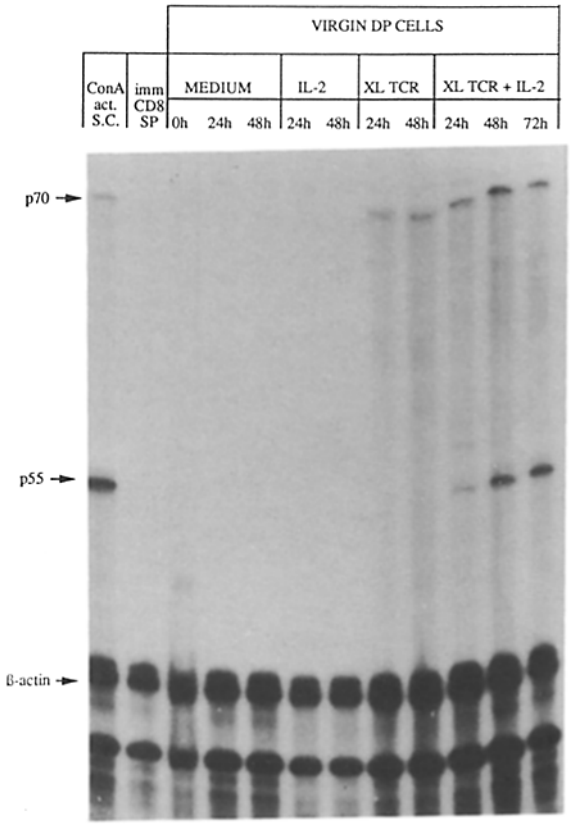

Figure 2. RNase protection analysis of IL-2R α and β chain mRNA transcripts. RNA was isolated from immature CD8 SP thymocytes (*imm CD8 SP*) and their progeny cultured under the conditions indicated. 5 μg cytoplasmic RNA/sample were analyzed by RNase protection for the presence of IL-2Rα (p55) and β (p70) transcripts. (→) Position of protected fragments.

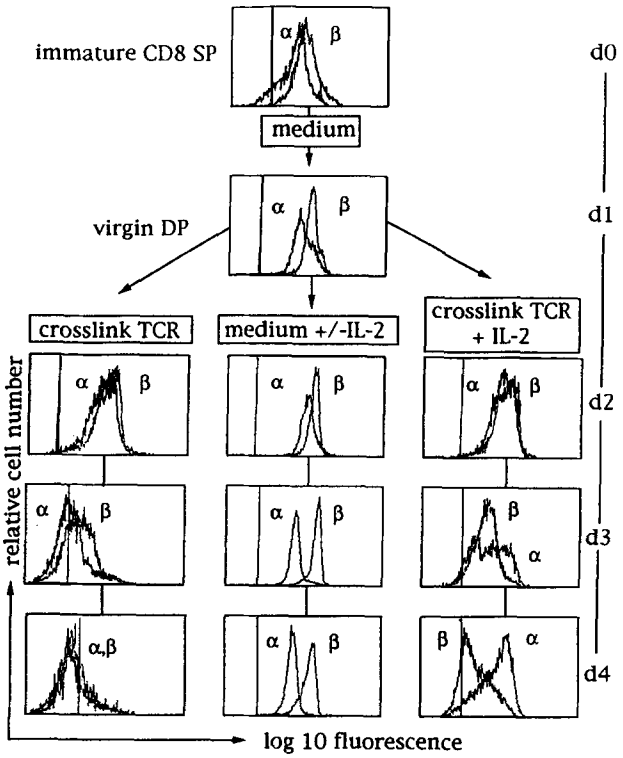

Figure 3. Modulation of CD8 α and β cell surface expression on DP thymocytes by TCR ligation and IL-2. Immature CD8 SP thymocytes were isolated and cultured as described in Fig. 1. At the times indicated, 5,000 cells were analyzed for the expression of CD8α using mAb OX-8, or for CD8α/β expression using mAb 341. (*Vertical line*) Upper limit of staining obtained with negative control mAb.

CD5, CD8 T cells generated in vitro were indistinguishable from those isolated from peripheral lymphoid organs (data not shown). A remarkable discrepancy, however, was detected using a novel mAb against CD8. This mAb, called 341, only reacts with rat CD8 molecules containing the β chain, and thus serves to distinguish CD8α homodimers from α/β heterodimers (19).

Fig. 3 illustrates the expression of CD8α and CD8α/β on thymocytes of the TCR-α/β lineage during passage from the immature CD8 SP through the DP compartment with and without TCR and/or IL-2 stimulation.

All CD8 immature thymocytes as well as their DP progeny expressed CD8 α and β chains (Fig. 3, *center*). TCR stimulation lead to downregulation of both chains of CD8 (Fig. 3, *left*) (and of CD4; 12 and Fig. 1). It is interesting that addition of IL-2 to TCR-stimulated DP cells (Fig. 3, *right*) resulted in a selective maintenance of CD8α expression, so that the resulting cells were mainly of the CD8-$\alpha^+/\beta^{low/-}$ phenotype.

In Fig. 4, these observations were extended to the mRNA level. When immature CD8 SP thymocytes or their DP progeny were analyzed for the presence of CD8α or CD8β transcripts by RNase protection assay, mRNA for both chains was detected. Moreover, a dramatic increase in both of these mRNA species was evident in thymocytes upon transition from the immature CD8 SP to the DP compartment, parallel to the slight increase in CD8 cell surface expression observed by immunofluorescence (Fig. 4). In contrast, cross-linking of the TCR by immobilized TCR-specific mAbs in the presence of IL-2 led to an almost complete loss of CD8β mRNA, whereas CD8 α chain mRNA was readily detectable. The reduction in CD8β mRNA level was even more complete than that of CD8β cell surface expression, suggesting that the low level of CD8β staining after 3 d of TCR plus IL-2 stimulation was due to residual and not newly synthesized protein. Thus, the change in CD8 isoform expression observed at the cell surface is accounted for by a change in the abundance of CD8α versus β transcripts, and therefore is likely to reflect a selective shut-off of CD8β transcription or of CD8β mRNA breakdown (30a).

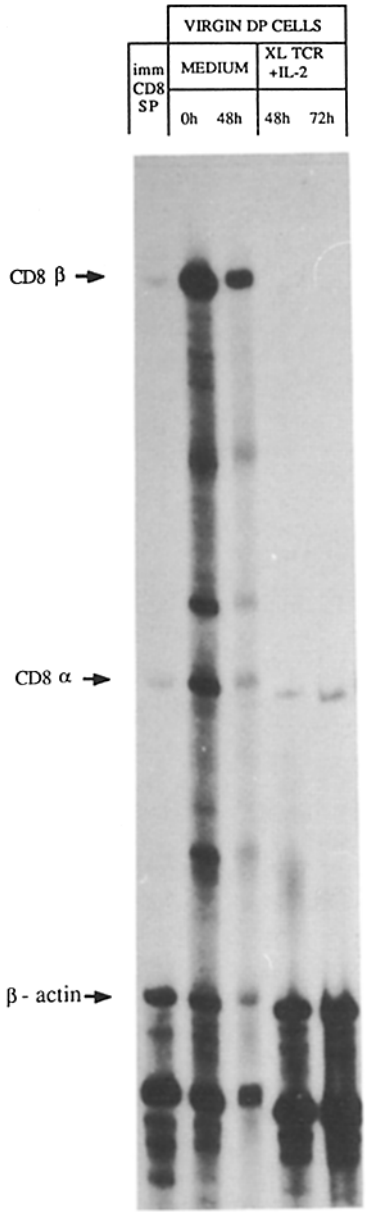

Figure 4. RNase protection analysis of CD8α and β mRNA transcripts. Cytoplasmic RNA isolated from immature CD8 SP thymocytes cultured as indicated were analyzed as described in Materials and Methods. (→) Positions of protected fragments.

Discussion

The present data show that TCR stimulation of immature DP thymocytes initiates a differentiation pathway that allows IL-2 to drive maturation of CD8α/α T cells via IL-2R containing the β but not the α chain. To our knowledge, this is the first description of functional IL-2R on thymocytes of the selectable DP/TCR^{low} compartment. Whether such α^-/β^+ "intermediate affinity" IL-2R (29) are actually induced in DP thymocytes in vivo as a consequence of TCR occupancy cannot be answered from our results but will be tested in TCR-transgenic mice where most DP cells bear receptors for positively selecting MHC antigens or for negatively selecting MHC–peptide complexes.

The CD8α/α phenotype of the T cells generated in vitro along the IL-2/IL-2Rβ pathway makes it unlikely that this system mimics intrathymic positive selection of CD8 T cells by self MHC class I, since MHC class I–restricted T cells almost exclusively express the CD8 heterodimer (19), and, at least in mice, mature in the absence of IL-2 (7). Rather, maturation of "CD8α only" T cells appears to be characteristic of extrathymic T cell development, since this subset is barely detectable in the thymus, lymph nodes, or spleens of euthymic mice or rats but is abundant among intestinal intraepithelial lymphocytes (IEL) and in lymphoid organs of athymic rats (19) and mice (31). It is interesting that generation of CD8α/α IEL in TCR-transgenic mice requires recognition of nominal antigen presented by self MHC (32), in contrast to positive selection of CD8α/β T cells, which are selected by self MHC in the absence of the nominal antigen. Thus, CD8α/α cells are generated in vivo under con-

ditions that negatively select CD8α/β T cell development. Ligation of the TCR by mAb in our system is likely to mimic recognition of antigen–MHC complexes that seems to suffice for negative selection (33), rather than the elusive and obviously more demanding signals that mediate positive selection. We thus hypothesize that DP thymocytes recognizing self antigens can be rescued by IL-2 into the CD8α/α compartment, and that this pathway may also be taken by immature T cell precursors outside the thymus.

Induction of CD8α homodimers on DN or CD4 SP T cells by T cell activation has been found in several species including the rat (19, 34, 35). It is thus possible that IL-2 not only rescued TCR-stimulated DP cells from cell death, but induced CD8α as a postmaturation event. The in vivo correlate of the differentiation pathway observed in vitro may thus be that of DP cells becoming DN followed by activation-induced expression of CD8α/α. Downregulation of CD4 and CD8 is indeed observed in cultures of rat (12) and mouse (33) DP thymocytes stimulated through the TCR in the absence of IL-2, and at least in rats, those CD4/8^{low} cells can still be rescued and converted to CD8α/α T cells by IL-2 (Fig. 1).

We have recently demonstrated that anti-CD2 stimulation converts rat DP thymocytes to functional DN T cells (36). Thus, rat DP thymocytes apparently respond to various modes of stimulation with downregulation of both coreceptors unless specific (but unknown) signals are provided that instruct the cell to maintain CD4 or CD8. Although its role in mainstream T cell maturation may be minor or redundant, IL-2 is able to provide such a signal in the generation of the CD8α/α subset from cells that once expressed both CD8 chains, and CD4.

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