Thymocytes Can Become Mature T Cells without Passing through the CD4$^+$CD8$^+$, Double-positive Stage

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Summary

T cells bearing the class II–restricted, DO–T cell receptor (TCR) are CD4$^+$ if their thymocyte precursors are positively selected on the class II protein, IA^d, but they are almost all CD4$^-$ after positive selection on a class II for which they have higher avidity, IA^b. DO-TCR$^+$ T cells mature in H-2^b mice lacking CD4. CD4$^-$ DO-TCR$^+$ T cells appear in H-2^b mice at the same rate as their CD4$^+$ counterparts appear in H-2^d animals, suggesting that the CD4$^-$ cells are not the product of some minor pathway of thymocyte development and selection. In H-2^b CD4 knock out mice expressing human CD2 under the control of the mouse CD4 promoter, mature DO-TCR$^+$ cells did not express human CD2. These results suggest that the CD4$^-$CD8$^-$, DO-TCR$^+$ mature T cells have developed without ever passing through the equivalent of a CD4$^+$, CD8$^+$ stage. The early expression of α/β receptors (TCRs) on thymocytes in TCR transgenic mice may allow maturation of this type. Passage through the equivalent of the CD4$^+$CD8$^+$, double-positive stage is not essential for differentiation of thymocytes into mature T cells.

In normal mice, most mature T cells bearing α/β TCRs express on their surfaces either CD4 or CD8 (1). Usually T cells bearing TCRs which are specific for antigenic peptides bound to class I MHC proteins bear also CD8, a protein which can engage a conserved site on class I MHC molecules (2, 3). Likewise, T cells bearing TCRs which are specific for antigenic peptides bound to class II MHC proteins bear also CD4, which can engage a conserved site on class II MHC (4–7). CD4 and CD8 probably contribute to T cell responses in several ways, among them the fact that their affinity for class I and class II, respectively, increases the overall avidity with which thymocytes and T cells can react with their targets (8–10).

Early thymocytes bear neither CD4 nor CD8 (11, 12). As the cells mature, however, both CD4 and CD8 appear on their surfaces and it is thought that it is at this stage that future expression of these proteins is determined, either by a random choice, followed by positive selection of cells bearing the protein appropriate to the specificity of their TCRs (13–16) or by instructed choice of the appropriate protein based on the specificity of their TCR (17, 18).

A few mature α/β TCR$^+$ T cells in normal animals bear neither CD4 nor CD8 (19, 20). Cells of this phenotype are, however, more frequent in diseased animals or in certain TCR transgenic mice. Many T cells in the autoimmune lpr/lpr strains of mice, for example, are CD4$^-$, CD8$^-$ double negative (DN[1], 21). These T cells have additional abnormalities, for example, they bear relatively low levels of TCR on their surfaces, they are B220$^+$, and they do not respond to normal T cell stimuli. Double-negative T cells have also been detected in mice expressing some transgenic TCRs. Cells of this phenotype are particularly likely to appear if the transgenic TCR reacts with some antigen in its host. For example, DN T cells were detected in male mice expressing a male antigen–specific TCR (22). Apart from the fact that they express neither CD4 nor CD8 the DN mature T cells in TCR transgenic mice are not like those in lpr/lpr animals. They do not bear B220 and in some circumstances they can respond to TCR engagement.

A number of investigators have tried to find out how these abnormal cells develop. The DN cells in lpr/lpr mice are thought to mature via a CD4$^+$CD8$^+$ stage, to be positively selected by reaction with class I MHC proteins, and, for some reason, then lose expression of both CD4 and CD8 (23, 24). In contrast, the maturational route of the DN cells in TCR transgenic mice is not well understood.

In this article we have examined the way in which cells of this phenotype appear in mice expressing a particular set

[1]*Abbreviations used in this paper:* cOVA, chicken OVA; DN, double negative; KO, knockout.

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of TCR transgenes, coding for a TCR specific for a chicken ovalbumin peptide (cOVA 323-339) bound to IA^d or IA^b which has also a low but appreciable reactivity for IA^b bound to an unknown mouse peptide. Cells bearing this TCR (DO-TCR) mature into $CD4^+$ cells in mice expressing IA^d, but into DN cells in mice expressing IA^b. Experiments show that the production of DN DO-TCR^+ cells in IA^b mice is an efficient event. Cells of this phenotype appear at the same rate as their $CD4^+$ counterparts do, in IA^d-expressing animals. The DN DO-TCR^- T cells appear in IA^b expressing mice even if CD4 is not expressed. We used a surrogate marker for CD4, human CD2 driven by a CD4 promoter, to show that these DN DO-TCR^+ T cells are not in the CD4-expressing lineage, and that it is likely that these cells mature without passing through the $CD4^+CD8^+$ stage of thymocyte development. Therefore this maturational stage must not be essential.

Materials and Methods

Mice. $H\text{-}2^{b,\ d,\ or\ k}$ mice expressing the DO-11.10 TCR transgene were obtained by breeding DO-TCR transgenic BALB/c mice (25) with C57BL/10, B10.D2nSn, or B10.BR mice obtained from The Jackson Laboratory, Bar Harbor, ME. $H\text{-}2^b$ or $H\text{-}2^d$, CD4-deficient (CD4 knockout [KO]) mice and CD4 KO mice expressing human CD2 driven by the mouse CD4 promoter were the kind gifts of Drs. Killeen (University of California, San Francisco) and Littman (New York University, New York) (10, 26–29). The DO-TCR transgenes were introduced into these animals by two generation crosses with DO-TCR transgenic BALB/c mice. DO-TCR^+ $H\text{-}2^b$ or $H\text{-}2^d$, RAG-2 deficient mice (RAG KO) were obtained in similar fashion using RAG KO animals kindly provided by Dr. Fred Alt (Children's Hospital, Harvard Medical School, Boston, MA) (30). Mice used in analysis were 4–12 wk old unless otherwise mentioned. All the mice were maintained in the specific pathogen-free Animal Care Facility at the National Jewish Center.

Production of Bone Marrow Chimeric Mice. C57BL/10 $H\text{-}2^b$ mice were obtained from The Jackson Laboratory. $H\text{-}2^b$ animals which do not express class I MHC proteins due to inactivation of the β2 microglobulin gene (class I KO) were the kind gifts of Drs. Koller and Smithies (University of North Carolina, Chapel Hill) (31). $H\text{-}2^b$ class II–deficient animals (Class II KO, 32) were purchased from GenPharm (Mountain View, CA). DO-TCR^+, $H\text{-}2^b$, CD4 KO donor and C57BL/10, class I KO, and class II KO recipient animals were treated with 0.4 ml rabbit anti–mouse thymocyte serum (Accurate Chemical and Scientific, Westbury, NY) to remove mature circulating T cells. 2 d later recipients were irradiated with 950 rads from a cesium source and given 5×10^6 bone marrow cells from donors intravenously. The mice were maintained on acidified chlorinated water.

Antibodies and Flow Cytometry. The following antibodies used for staining were purified and conjugated in our laboratory: biotinylated-KJ1 (anti-DO-TCR idiotype, 33), biotinylated-H57-597 (anti-TCR Cβ), biotinylated-F23.2 (anti-TCR V8.2), FITC-conjugated GK1.5 (anti-CD4) were purified and conjugated in the laboratory; anti-CD4-PE, anti-CD8-FITC, anti-B220-PE, anti-H-2 D^d-FITC, anti-H-2 K^b-PE, anti–human CD2-FITC and streptavidin-Cychrome were purchased from PharMingen (San Diego, CA).

Single cell suspensions obtained from thymus and LN (pooled from inguinal, brachial, and axillary nodes), were filtered through nylon mesh (Falcon Labware, Becton Dickinson and Co., Franklin Lakes, NJ), washed in balanced salts solution (BSS) and stained with antibodies at $1\text{–}3 \times 10^7$cells/ml in staining buffer (BSS, 0.1% sodium azide, 2% fetal bovine serum). Two- or three-color staining was performed as follows. Cells were incubated with 10% normal mouse serum and a 10% culture supernatant of the anti-Fc receptor antibody 24.G2 (34) to block nonspecific binding of the staining antibodies. Primary antibodies were added to the cells in the presence of blocking agents, incubated for 30 min at room temperature, and the cells were washed three times before addition of the second reagent, streptavidin-Cychrome or streptavidin-PE. Cells were incubated with the second reagents at 4°C for 30 min. At least 10^4 cells were assessed on a FACScan® (Becton Dickinson and Co., San Jose, CA) and analyzed using the PC-LYSIS program (Becton Dickinson and Co.).

Proliferation Assays. Spleens and peripheral lymph nodes were teased through nylon mesh, and spleen cells were then subjected to ammonium chloride solution to lyse red blood cells. After one wash with BSS, the cells were maintained in DMEM supplemented with 10% FBS, sodium bicarbonate, antibiotics, 2-ME, sodium pyruvate, and nonessential amino acids. Cells were titrated in triplicate into the wells of 96-well flat-bottomed plates (Falcon Labware, Becton Dickinson and Co.) containing 5×10^5 irradiated (3,000 rad with a Cs source) splenocytes/well. The cOVA 323-339 was used as antigen at 5 μM. Antibodies used for stimulation were fixed to Immulon 4, 96-well flat-bottomed plates (Dynatech Laboratories, Chantilly, VA) at a concentration of 5–25 μg/ml during an overnight incubation at 4°C. Nonattached antibodies were removed by five vigorous washes with BSS before cell addition. Cells were cultured in these wells for 72 h at 37°C and incubated with 1 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) per well, during the last 12–16 h of incubation. At the end of the incubation, cells were harvested on a Harvester 96 Mach III M (Tomtec, Orange, CT) and the incorporation was determined on a Microbeta PLUS liquid scintillation counter (Wallac, Turku, Finland).

Southern Blot Analysis. The methylation status of the CD8 genes in thymocytes, T cells, and liver cells was examined as previously described (35). DNA was digested with the indicated restriction enzymes, blotted onto nitrocellulose filters, and probed with a PCR-amplified PstI-EcoRI DNA fragment of the Lyt-2 gene (36). $CD4^+CD8^-$, $CD4^-CD8^+$, and $CD4^-CD8^-$ T cells were isolated using affinity columns (Biotex Laboratories Inc., Edmonton, Canada).

Results

Cells Expressing the DO-TCR Behave Differently in Mice of Different MHC Types. T hybridoma cells bearing the DO-TCR and CD4 react well with cOVA 323–339 bound to IA^b or IA^d and have no detectable ability to react with either IA^d or IA^b in the absence of the ovalbumin peptide (37). $CD4^+$ T cells bearing the DO-TCR which have matured in $H\text{-}2^d$ animals do, however, proliferate after transfer into $H\text{-}2^b$ animals (data not shown), suggesting that the DO-TCR may have a detectable affinity for IA^b bound to one or more endogenous mouse peptides. DO-TCR–bearing cells react with the antiidiotypic antibody, KJ-1 (33). This antibody was used to demonstrate that the DO-

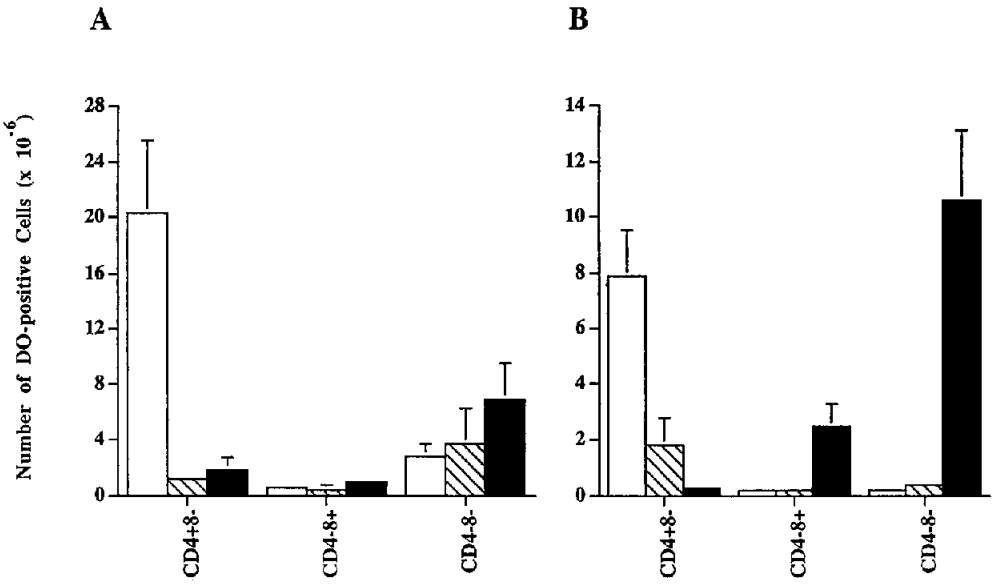

Figure 1. Phenotype of mature thymocytes and T cells in DO-TCR–transgenic mice with various H-2 haplotypes. Thymus and LN cells were isolated from animals of different MHC haplotypes and stained for surface expression of CD4, CD8, and the DO-TCR as described in Materials and Methods. Shown are the means and standard errors of the numbers of cells bearing high levels of the DO-TCR which were $CD4^+CD8^-$; $CD4^-CD8^+$; or DN in: (*A*) the thymuses and (*B*) the LN of $H\text{-}2^d$ (□); $H\text{-}2^k$ (▨); and $H\text{-}2^b$ (■) mice.

TCR is expressed predominantly on $CD4^+$ T cells in DO-TCR transgenic $H\text{-}2^d$ mice (25).

To study the influence of various H-2 molecules on the development of thymocytes, the DO-TCR transgenes were crossed into mice of different H-2 haplotypes on the C57BL/10 background. The numbers of $CD4^+$ DO-TCR^+ cells were compared between the different types of mice (Fig. 1, *A* and *B*). We focused our studies on the cells that bore high levels of the DO-TCR, indicated by good staining with KJ1 (DO-TCR^{hi}), because these cells bore the DO-TCR and did not bear appreciable levels of any other TCR, as indicated by the fact that they did not stain better with the biotinylated anti-Cβ antibody, Ham-597, than they stained with the biotinylated antiidiotype antibody, KJ1, two antibodies known to stain T cell hybridomas bearing only the DO-TCR equally well (data not shown). Positive and negative selection of these DO-TCR^{hi} cells must therefore be governed by the DO-TCR itself, and not some other TCR of unknown specificity.

DO-TCR transgenic mice which were homozygous for $H\text{-}2^k$ or $H\text{-}2^b$ had many fewer $CD4^+$ DO-TCR^{hi} cells than $H\text{-}2^d$ mice had (Fig. 1, *A* and *B*). This suggests that thymocytes bearing DO receptors only are either not positively selected or are deleted in animals homozygous for H-2 k or b. It has previously been shown that T cells bearing the DO-TCR and CD4 are deleted by $H\text{-}2^b$ (Murphy, K., personal communication). To confirm this, and find out why DO-TCR^+ T cells failed to appear in $H\text{-}2^k$ mice, we examined the fate of these cells in F1, $H\text{-}2^{kxd}$, or $H\text{-}2^{bxd}$ animals. The numbers of $CD4^+$ DO-TCR^{hi} cells in the thymuses and lymph nodes of F1 $H\text{-}2^{kxd}$ mice were similar to those in $H\text{-}2^d$ mice, whereas F1 $H\text{-}2^{bxd}$ animals had very few $CD4^+$ DO-TCR^{hi} cells in their thymuses or lymph nodes by comparison with $H\text{-}2^d$ animals (Fig. 2, *A* and *B*). These results indicated that thymocytes bearing the DO receptor are not deleted by molecules associated with $H\text{-}2^k$, and therefore the lack of $CD4^+$ DO-TCR^{hi} cells in animals expressing $H\text{-}2^k$ is due to lack of positive selection. By

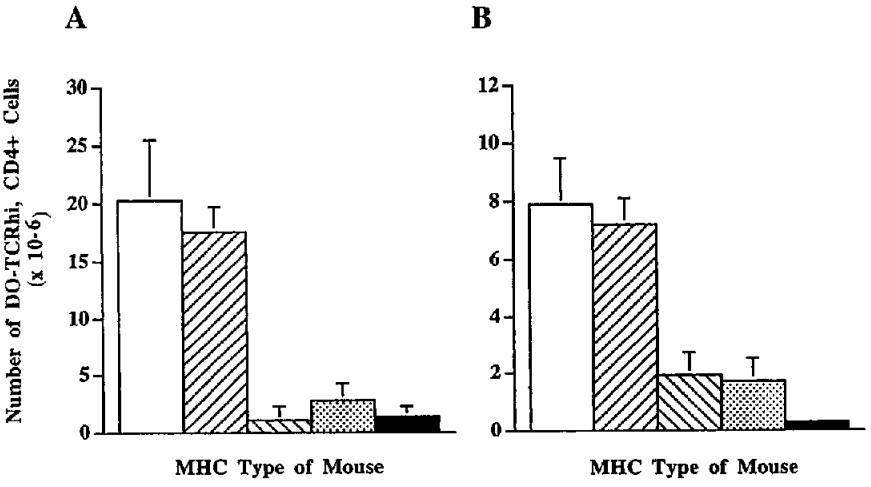

Figure 2. $H\text{-}2^b$ deletes and $H\text{-}2^k$ does not positively select $CD4^+$ T cells bearing the DO-TCR. Thymus and LN cells were isolated from animals of different MHC haplotypes and stained as described in the legend to Fig. 1. Shown are the means and standard errors of the numbers of cells bearing high levels of the DO-TCR and CD4 in (*A*) the thymuses and (*B*) the LN of $H\text{-}2^d$ (□); (kxd)F1 (▨); $H\text{-}2^k$ (▨); (bxd)F1 (▨); and $H\text{-}2^b$ (■) mice.

Table 1. *Cellularity of DO-TCR Transgenic Thymuses of Different MHC Types*

	MHC Type		
	H-2^d	H-2^b	H-2^k
Cells/Thymus ($\times 10^{-8}$)	1.6 ± 0.35	1.3 ± 0.3	3.5 ± 0.45

Thymuses were removed from 4- to 8-wk-old mice. Results shown are mean ± SE.

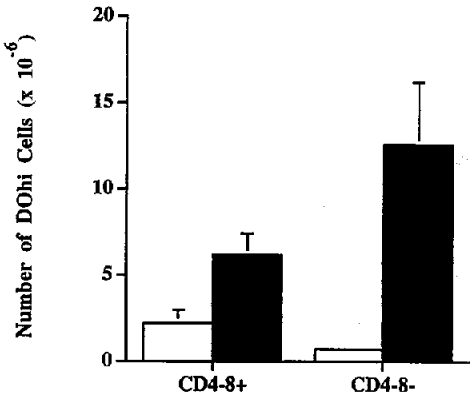

Figure 3. Relatively strong interactions between TCR and class II MHC allow positive selection in the absence of CD4. The DO-TCR transgene was bred into CD4 KO mice expressing H-2^b (■) or H-2^d (□) as described in Materials and Methods. Shown are the means and standard errors of the numbers of cells in the LN of each mouse bearing high levels of the DO-TCR and CD8, not CD4, or neither CD4 nor CD8.

contrast, expression of H-2^b, even in heterozygotes, caused the disappearance of CD4$^+$ DO-TCR^{hi} cells and therefore the absence of such cells in H-2^b homozygotes is due to negative selection.

In spite of the fact that H-2^b expression is tolerogenic for T cells bearing the DO-TCR, it is interesting to note that the thymus cellularity of H-2^b adult mice was not reduced by comparison with that of H-2^d animals (Table 1). Perhaps this is because the DO-TCR does not react very strongly with H-2^b, as witnessed by the fact that mature DO-TCR–bearing T cell hybridomas do not secrete IL-2 in response to H-2^b (37) although, as noted above, CD4$^+$ DO-TCR$^+$ T cells can respond to H-2^b in vivo. On the other hand, the thymuses of H-2^k DO-TCR transgenic mice contain many more thymocytes than do the other thymuses. This result suggests that, by some unknown mechanism, positively selected mature thymocytes control and reduce the numbers of precursor cells in the organ.

In summary, mice homozygous for H-2 d, k, or b provide positively selecting, nonselecting, and deleting backgrounds, respectively, for CD4$^+$CD8$^-$ thymocytes expressing high levels of the DO-TCR. These mice were used to examine these phenomena in greater detail as described below.

Expression of H-2^b Causes the Appearance of DO-TCR^{hi} CD4$^-$ Mature T Cells. Many of the LN T cells in H-2^b mice bore high levels of the DO-TCR and were either DN or CD4$^-$CD8$^+$ (Fig. 1 *B*). These cells stained equivalently with the anti-DO idiotypic antibody, KJ1, and antibody to TCR Cβ (data not shown), therefore the cells probably bore a single TCR that encoded by the DO-TCR transgene. The results suggested that interaction between this TCR and IA^b in the thymus allowed positive selection of cells expressing neither CD4 nor CD8 or of cells expressing only CD8.

Several experiments were done to check that this was indeed the case. In the first, the DO-TCR transgenes were crossed into mice that could not express CD4 (CD4 KO animals, 26, 27). As shown in Fig. 3, very few DO-TCR^{hi} T cells of any CD4/8 phenotype appeared in the LN of

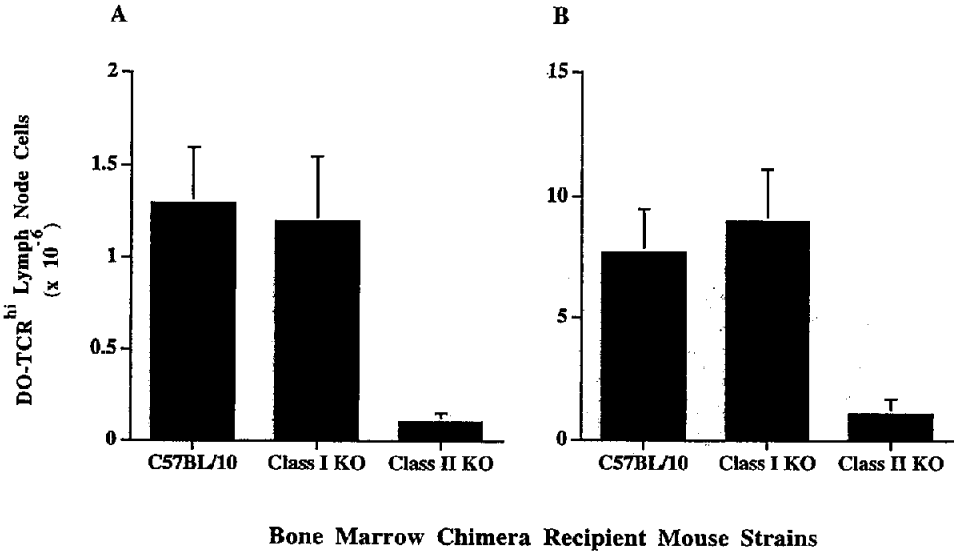

Figure 4. Cells bearing the DO-TCR are selected by reaction with class II MHC regardless of their subsequent phenotype. Normal C57BL/10 or β2 microglobulin–deficient (class I KO) or class II–deficient (class II KO) mice were irradiated and reconstituted with bone marrow from DO-TCR transgenic mice as described in Materials and Methods. 6 wk after irradiation and bone marrow reconstitution, LN T cells in the recipient mice were analyzed for expression of high levels of the DO-TCR and CD4 and CD8. Shown are the means and standard errors of the numbers of DO-TCR^{hi} cells/LN of three recipient animals which were: (*A*) CD4$^-$CD8$^+$ or (*B*) DN.

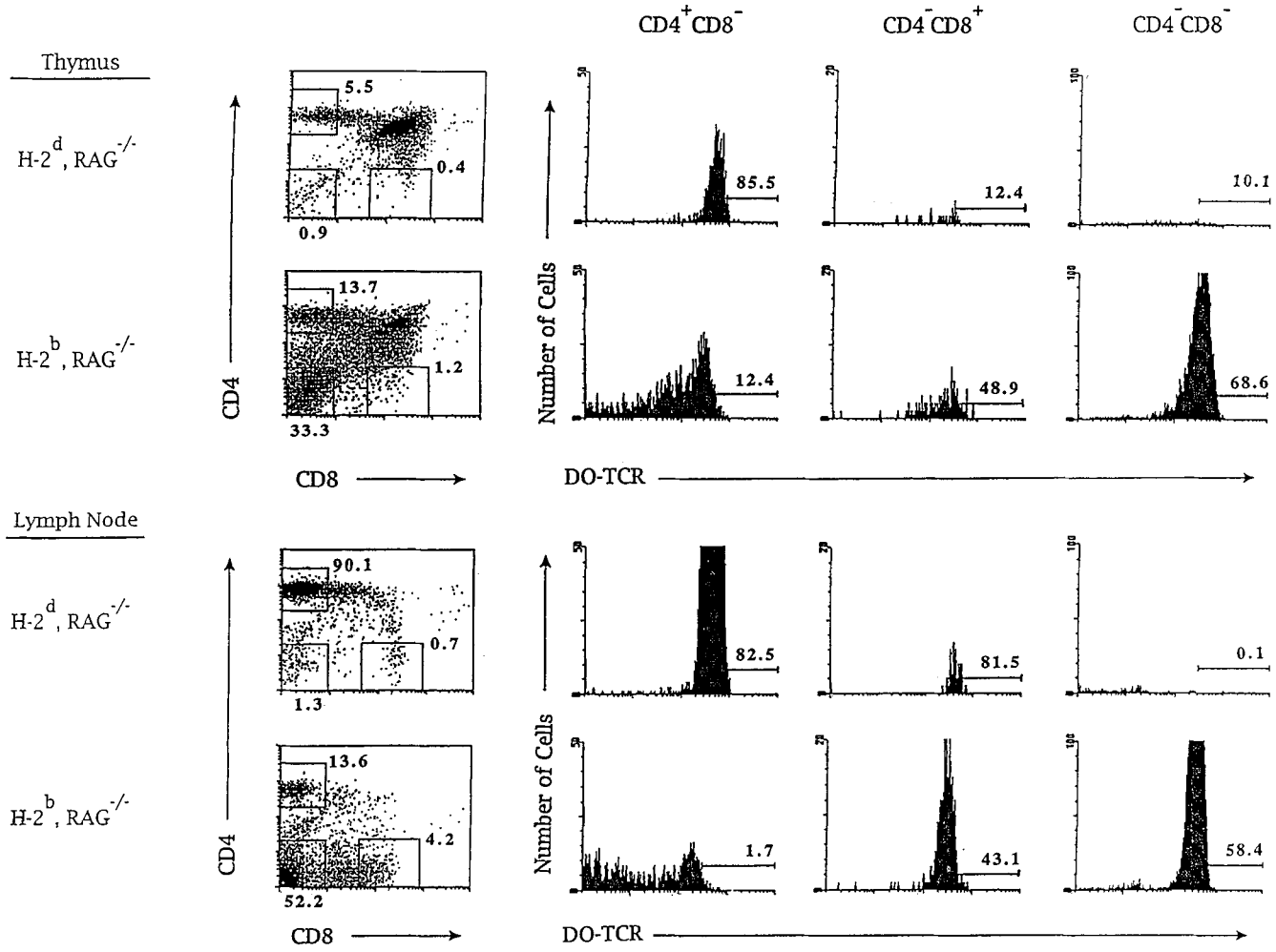

Figure 5. The phenotypes of thymocytes and T cells in mice that express only the DO-TCR. The DO-TCR transgene was bred into RAG KO mice expressing H-2^b or H-2^d and thymocytes and LN from these animals were analyzed for expression of CD4, CD8, and the DO-TCR as described in Materials and Methods. Shown are the dot plots of CD4 vs CD8 staining of thymus and LN cells from H-2^b or H-2^d RAG KO mice. The percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, or DN cells in each of these populations is indicated on the dot plot next to the appropriate gated region. The adjacent histograms illustrate the levels of DO-TCR stained on each of the gated populations. The numbers on the histograms represent the percentage of cells in the region bearing high levels of the DO-TCR. The average total number of cells are: 2–3 × 10^7 in thymus and 1.5–3 × 10^6 in LN of both H-2^d and H-2^b animals.

CD4 KO, H-2^d mice. On the other hand, CD4 KO, H-2^b animals contained many cells lacking CD4 but bearing high levels of the DO-TCR without or with coexpression of CD8. These results show that CD4 expression is not required at all for positive selection of thymocytes bearing the DO-TCR in H-2^b thymuses.

It was possible that these cells were not selected by reaction with the class II protein expressed in H-2^b, IA^b, but rather by reaction with one of the classical or nonclassical class I proteins coded by this haplotype. To check this, DO-TCR transgenic thymocytes were tested for their ability to mature in thymuses that could not express either the class I or the class II MHC proteins of H-2^b. Thus, bone marrow cells from CD4 KO, DO-TCR$^+$, H-2^b mice were used to reconstitute lethally irradiated H-2^b mice that could not express either β2-microglobulin (class I KO, 31) or the β chain of I-A^b (class II KO, 32). As shown in Fig. 4, T cells bearing high levels of the DO-TCR with or without CD8 appeared in good numbers in wild-type control animals, and in class I KO mice, but were few in number in class II KO mice. Therefore, in H-2^b animals, thymocytes bearing the DO-TCR are positively selected by reaction with I-A^b but not with I-A^d and this event does not require expression of CD4.

Finally, in spite of the fact that the cells assessed in these experiments bore high levels of the DO-TCR, it remained possible that some of these cells had rearranged genes for other TCR α and/or β chains, and that low level expression of these other genes was affecting our results. To control for this, the DO-TCR transgenes were bred into H-2^d or H-2^b mice lacking expression of RAG-2 gene (RAG KO, 30). These mice cannot rearrange and express other TCR

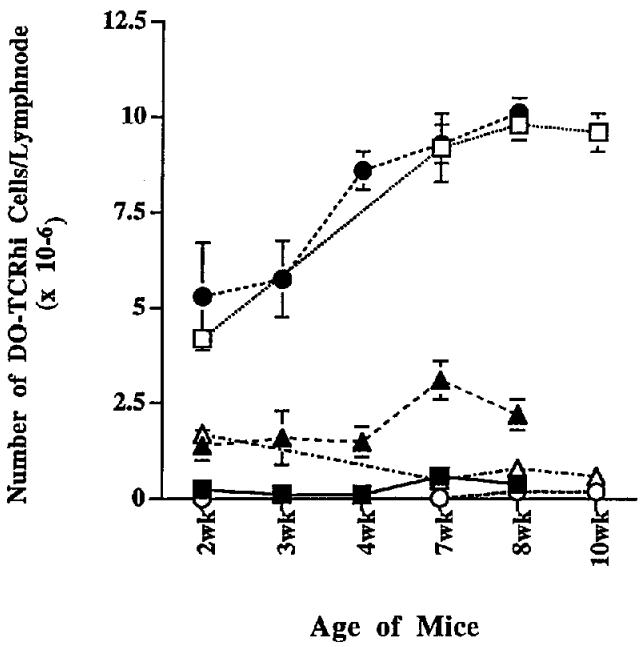

Figure 6. DN T cells bearing the DO-TCR accumulate at high rate in the lymph nodes of H-2^b mice. The numbers of cells bearing high levels of the DO-TCR and various combinations of CD4 and CD8 in the LN of DO-TCR transgenic mice of various ages were measured using flow cytometry. Shown are the means and standard errors of these numbers derived from at least three mice of each age. $CD4^-CD8^-$ H-2^b (■); $CD4^+CD8^-$ H-2^d (□); $CD4^-CD8^+$ H-2^b (▲); $CD4^-CD8^+$ H-2^d (△); DN H-2^b (●); DN H-2^d (○).

genes. Thymocytes and LN cells from these mice were examined for the phenotypes of the DO-TCR–bearing cells.

Since T cells bearing the DO-TCR and CD4 are positively selected by reaction with I-A^d, we were not surprised to find that $CD4^+$ DO-TCR^+ cells were well represented in the thymuses and LN of H-2^d, RAG KO animals (Fig. 5). However, it is worth noting that even in these H-2^d mice a few thymocytes and LN cells bore high levels of the DO-TCR and CD8, not CD4.

As the results described above had led us to expect, not only the LN but also the thymuses of RAG KO H-2^b animals contained many cells bearing high levels of the DO-TCR and neither CD4 nor CD8. To a lesser extent, these animals also contained DO-TCR^{hi} thymocytes and T cells bearing CD8 and not CD4. Almost all of the $CD4^+$ cells in these animals bore reduced levels of the DO-TCR.

Collectively, these results show that thymocytes bearing the DO-TCR are selected much more efficiently on IA^d if they coexpress CD4. By contrast, selection of these thymocytes on I-A^b does not require coexpression of CD4 and, in fact, most of the cells selected by reaction between the DO-TCR and IA^b but not IA^d do not express CD4 or, indeed, CD8. Selection on IA^b and, to a lesser extent, IA^d, does create some mature cells bearing the "inappropriate" accessory protein, CD8. Thus the MHC protein with which the TCR reacts during positive selection does not always determine the CD4 or CD8 phenotype of the T cell which is produced.

DN DO-TCR^{hi} Cells Are Produced by an Active Process. The results described above show that DN DO-TCR^{hi} T cells are the major T cell population in DO-TCR transgenic H-2^b mice. However, the data do not indicate how these cells are generated. For example, the data do not indicate whether these cells accumulate slowly as a result of a minor thymus maturation pathway, or rapidly, driven by a dominant route. Two sets of data bear on the matter. The first set derives from the data in Fig. 5 which indicate that not only periphery tissues, but also the thymuses of H-2^b mice contain increased numbers of DN DO-TCR^{hi} cells by comparison with lymph nodes and thymuses from H-2^d mice. Secondly, we compared the numbers of cells of different subsets present in the LN of mice at different ages (Fig. 6). The results show that the DN DO-TCR^{hi} cells in the periphery of H-2^b mice accumulate at the same rate as their $CD4^+$ counterparts in H-2^d animals. Perhaps appearance of these cells is due to directed down-regulation of CD4 gene expression caused by the strength of the signal generated by reaction of the DO-TCR with IA^b in the thymus. Alternatively, the DN mature T cells might be selected randomly but expand rapidly to fill the void created by the absence of other T cells. Lastly, it is possible that these cells may be selected at the DN stage of thymocyte development without ever becoming $CD4^+CD8^+$ cells.

DO-TCR^+ Mature T Cells Develop without Passing through the Equivalent of a $CD4^+$ $CD8^+$ Stage. Three different protocols were used to test the idea that DO-TCR^+ T cells might mature without passing through a CD8-expressing stage. In the first, we injected DO-TCR^+, RAG-$2^{-/-}$ H-2^{bd} newborn mice with anti-CD8 antibodies to see whether development of these cells was blocked as predicted by previous studies on chimeric mice (38). The results showed that the anti-CD8 antibody did not interfere with the development of these cells (data not shown). However, it did not block the appearance of $CD4^+$ T cells in control animals, so this experiment was inconclusive.

We have previously shown that the methylation status of the CD8 gene indicates whether or not T cells have passed through a stage during which CD8 is expressed. In normal mice, restriction enzyme analysis reveals that the CD8 gene is undermethylated in $CD4^+$ mature T cells, indicating that these cells are derived from precursors that expressed CD8 (35). We applied this technique to DNA from the DN DO-TCR^+ T cells in H-2^b mice. As shown in Fig. 7, BamHI digestion alone yields an 8-kb band for the CD8 gene in DNA from all tissues. Double digestion with BamHI and MspI, a restriction enzyme that is not sensitive to the methylation status of the DNA it is digesting, yielded, from all tissues tested, a dominant 2.8-kb band which hybridized to the CD8 probe. Double digestion with BamHI and HpaII, a methylation-sensitive isoschizomer of MspI, yielded different results, however. Liver DNA gave a major 8-kb band after such digestion, indicating that the HpaII sites in the CD8 gene in liver DNA are methylated. By contrast, DNA from $CD8^+$ mature T cells yielded a major band at 2.8 kb with a minor band at 4.3 kb and faint bands at 8 and 3.4 kb, indicating almost complete

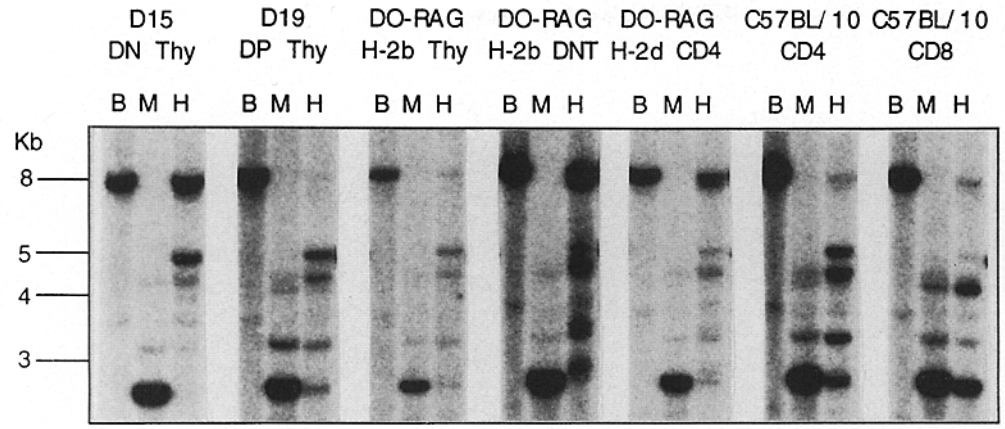

Figure 7. The methylation pattern of the CD8 gene in $CD8^-$ transgenic T cells is different from that of normal $CD4^+$ T cells. The methylation status of the CD8 genes of various cell populations was determined by restriction enzyme digestion and Southern analysis as described in Materials and Methods. Thymocytes from C57Bl/10 fetal mice of embryonic ages day 15 and 19 were used as sources of DN and $CD4^+CD8^+$ cells, respectively.

absence of methylation of the HpaII sites in the CD8 gene in these cells. The pattern of the CD8 genes from $CD4^+$ T cells from normal mice showed that these genes were somewhat more methylated than those of $CD8^+$ T cells, since they yielded bands of equal intensity at 5.0, 4.3, 3.4, and 2.8 kb. The digestion patterns of the CD8 genes from the DN DO-TCR^+ T cells from H-2^b mice, and from the $CD4^+$ DO-TCR^+ T cells from H-2^d mice were similar to each other with a major band at 8 kb and minor bands at 5.0, 4.3, 3.4, and 2.8 kb. This pattern is intermediate between that of DN day 15 immature thymocytes, which have never expressed CD8, and that of $CD4^+CD8^+$ immature thymocytes. These results suggest that the mature DO-TCR^+ T cells may have spent little or no time as immature $CD4^+CD8^+$ thymocytes, whether or not they eventually express CD4.

Because neither of the experimental approaches described above was conclusive, we developed a third protocol to test the idea that in H-2^b animals DO-TCR^+ T cells might appear without passing through the double-positive stage of thymocyte existence. To do this mice were created which expressed the DO-TCR and human CD2 as a surrogate for mouse CD4, that is, the mice did not express CD4 and carried a transgene for human CD2 driven by the mouse CD4 promoter (CD4 KO, $huCD2^+$, 26–29). We reasoned that expression of human CD2 on T cells in these mice should indicate whether or not these cells had differentiated into the CD4-expressing pathway without the disadvantage that mouse CD4 engagement might deliver an inhibitory or tolerizing signal to the cells. It is unlikely that the huCD2 itself would deliver such a signal since expression of human CD2 does not affect the overall developmental pathway of thymocytes (39). Moreover, all the thymocytes in these studies already expressed high levels of mouse CD2 (40) which would presumably already be delivering any signal mediated by this molecule (41).

As has previously been reported, expression of huCD2 driven by a CD4 promoter had no detectable effect on the development of thymocytes in CD4 KO animals (29). Most of the mature T cells which appear in such mice are $CD8^-$ and very few of the peripheral T cells are $huCD2^+$ (Fig. 8 *A*). Likewise, the presence of the huCD2 transgene had no effect on the phenotype of the T cells which matured in DO-TCR transgenic, H-2^b mice. The DO-TCR^+ mature T cells in H-2^b mice expressing huCD2 as a surrogate for mouse CD4 had the same phenotype as their counterparts in mice lacking the huCD2 transgene (Fig. 8 *B*). That is, most of the DO-TCR^+ T cells were $huCD2^-$, $CD8^-$, and, of course, $CD4^-$. A few of the DO-TCR^+ T cells did express huCD2, so expression of this gene was not lethal to cells of this type, but the vast majority of the mature DO-TCR^+ T cells in these mice were $huCD2^-$. These results demonstrated that the phenotype of the CD4 DO-TCR^+ T cells in IA^b mice was not due to some reaction driven by CD4 itself. Rather, it seemed that these cells could exist without induction of CD4 at all.

The DO-TCR^+ Cells Positively Selected by Reaction with IA^b Are Functional. We wished to find out whether the unusual $CD4^-$ DO-TCR^+ T cells in H-2^b animals cells could respond normally to stimulation via their TCRs. Therefore T cells from DO-TCR transgenic, H-2^b, and H-2^d mice were titrated for their ability to respond to syngeneic APC and cOVA 323-339 or to plate-bound anti-TCR antibodies. T cells from H-2^b mice responded to the OVA peptide plus IA^b (Fig. 9). Their response was not as good as that of T cells from H-2^d animals, however. This was partly because IA^b does not present cOVA 323-339 to the DO-TCR as well as IA^d does (data not shown), and partly because the response of DO-TCR^+ T cells to the peptide is augmented by coexpression of CD4 (8) and the DO-TCR^+ T cells from H-2^b mice lack CD4. The T cells responded equally well to anti-CD3 or anti-TCR Cβ antibodies, regardless of their strain of origin (Fig. 9), therefore there is no indication that the $CD4^-$ DO-TCR^+ T cells from H-2^b animals are not functional.

Discussion

Some years ago it was suggested that the fate of thymocytes is determined by the affinity with which their TCRs react with peptide/MHC complexes presented in the thymus cortex. High affinity reactions would cause the thymocyte involved to die, intermediate affinity reactions would lead to positive selection, and very low or undetect-

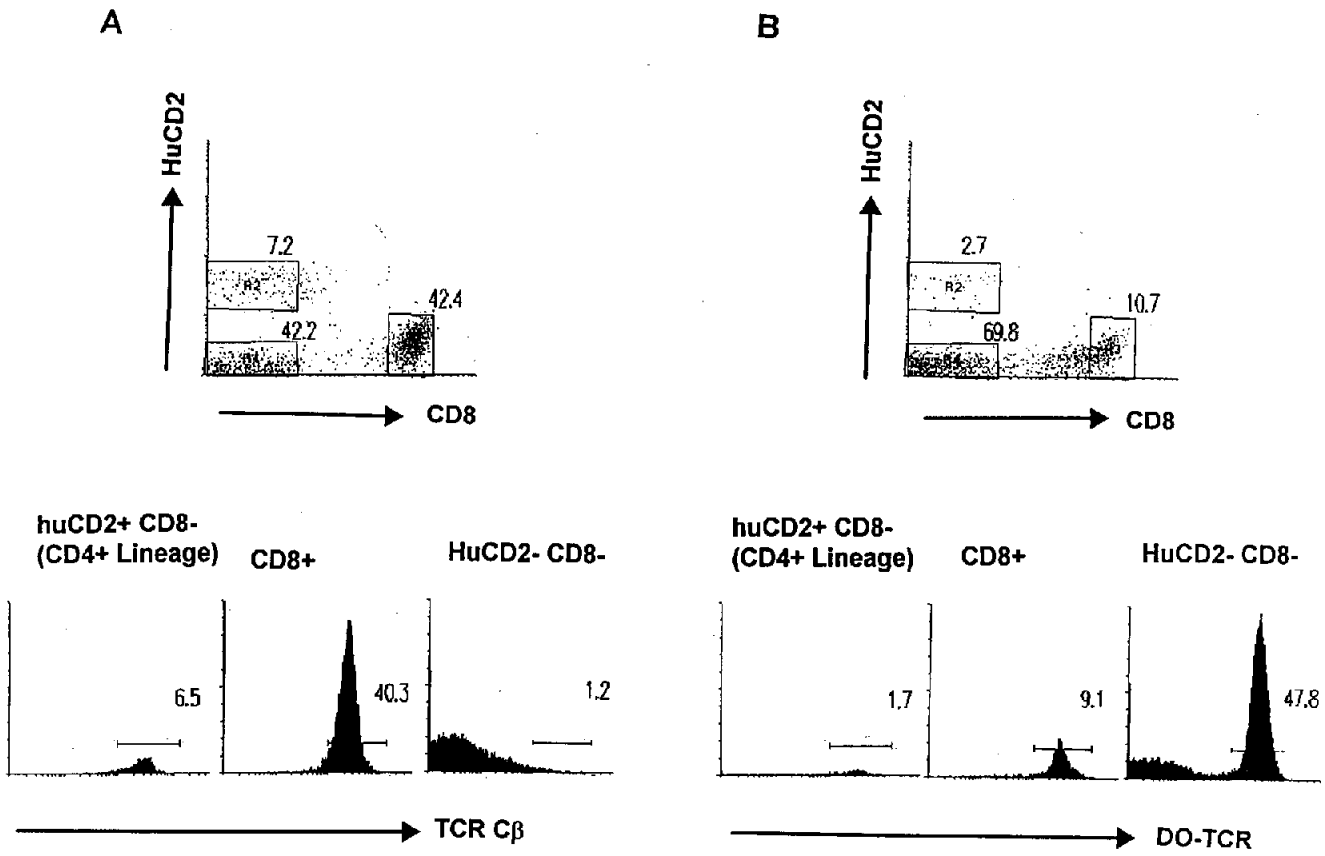

Figure 8. The DN DO-TCR$^+$ T cells that mature in IA^b mice probably do not pass through a CD4$^+$CD8$^+$ stage in the thymus. The DO-TCR transgene was crossed into H-2^b mice which lacked expression of CD4 and contained a human CD2 transgene driven by the mouse CD4 promoter and enhancer. LN T cells from DO-TCR nontransgenic (*A*) and transgenic (*B*) 6–8-wk-old mice were purified and stained for expression of human CD2, mouse CD8, and TCR Cβ (*left*) or the DO-TCR (*right*). The upper dot plots show the profile of human CD2 vs CD8 expression. The lower histograms show the levels of expression of TCR Cβ or the DO-TCR on LN cells that were, from left to right in each panel, human CD2$^+$, CD8$^-$; human CD2$^-$CD8$^+$; human CD2$^-$CD8$^-$. Numbers on the dot plots show the percentage of total LN cells which fell into the indicated regions. Numbers on the histograms show the percentage of total LN cells which fall into the indicated C or DO-TCR staining gates.

able affinity would leave the thymocyte to die of neglect (42). Recent experiments have supported this hypothesis, with the additional caveat that positive selection may depend on the avidity of the reaction between the thymocyte and selecting stromal cells, rather than the affinity of the TCR for peptide/MHC (43–45). The avidity of the interaction is crucial because it affects the stability of TCR engagement by ligand, and hence the strength of signal transduction through the TCR. It may also be important because thymocyte surface proteins such as CD4 and CD8 which increase avidity by binding to ligands on stromal cells may transduce signals themselves which contribute to the selecting signal (46–48). However, "accessory protein" signals, such as those transduced through CD4, may not be essential for positive selection (9, 10, 49).

The work described in this paper certainly supports these ideas. IA^d allowed the positive selection of thymocytes bearing the DO-TCR only if the thymocytes bore CD4 since selection did not occur in CD4 KO, H-2^d mice. On the other hand, IA^b, which probably reacts with the DO-TCR with higher affinity than IA^d does, did drive positive selection of DO-TCR$^+$ thymocytes whether or not CD4 was expressed. Since signals transduced through CD4 are therefore not essential for thymocyte-positive selection, one interpretation of this result is that the role of CD4 and/or CD8 in positive selection is not to transduce signals but rather to increase the avidity with which thymocytes interact with stromal cells and therefore effectively increase the signal transduced through TCRs. Alternatively, however, signals transduced through CD4 may contribute to the positively selecting signal delivered to the DO-TCR–bearing thymocyte selected on the weaker ligand, IA^d.

How do the CD4$^-$, class II–restricted T cells arise, in such large numbers in IA^b mice and even, admittedly in low numbers, in IA^d animals? In H-2^b animals most of the mature T cells bearing the DO-TCR express neither CD4 nor CD8. The phenotype of these cells is therefore somewhat reminiscent of that of the DN cells which appear in other, special, situations. However, we do not think that these cells are analogous to most of these others. For example, DN T cells appear in Fas$^-$ or Fas ligand–deficient animals. However the cells that appear in Fas-deficient mice

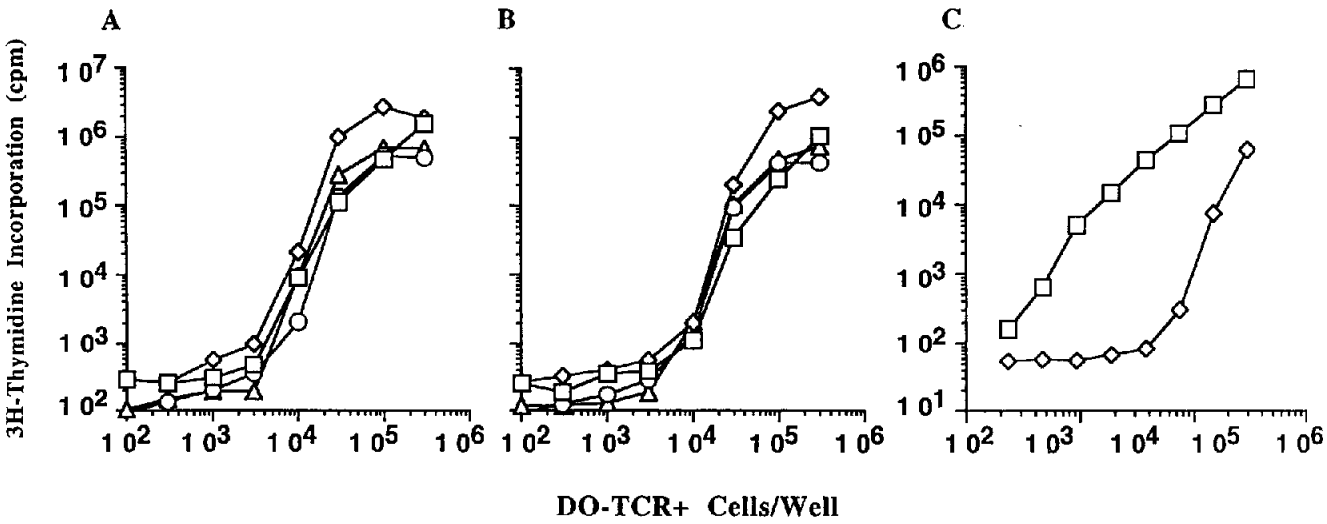

Figure 9. The DN DO-TCR$^+$ T cells are functional. T cells were purified from the LN of DO-TCR transgenic mice and titrated for their ability to respond to anti-CD3 antibody (*A*) or anti-Cβ antibody (*B*) coated onto plastic or to respond to specific antigen, cOVA 323-339, at 20 μg/ml (*C*) as described in Materials and Methods. 3 d later the cultures were assayed for thymidine incorporation for response. The results shown are the average incorporations in triplicate wells from one of two similar experiments. The genotypes of the donors were as follows: H-2^d (□); H-2^b (◇); H-2^d, RAG KO (○); H-2^b, RAG KO (△).

are B220$^+$ and most are selected on class I (50–52). Neither of these properties characterizes the DN DO-TCR$^+$ cells which arise in IA^b-expressing mice. Their requirement for selection on class II also indicates that the DN DO-TCR$^+$ cells in H-2^b mice are not related to the recently described DN cells specific for nonclassical class I–presented antigens (28, 53). The DN DO-TCR$^+$ T cells may have matured by the pathway that governs γ/δ T cell development. Unlike γ/δ cells, however, these cells require a specific class II MHC for positive selection since they do not appear in H-2^k mice, nor in animals that lack class II. Finally, von Boehmer et al. have reported that DN T cells mature in mice transgenic for a TCR specific for an HY peptide bound to D^b (22). These T cells did not, however, require positive selection on their expected MHC selecting protein, D^b, so these cells do not appear to be related to the cells described in this paper, which do require positive selection on a specific class II molecule, IA^b.

Thus the DN DO-TCR$^+$ cells produced in H-2^b animals appear to be normal, class II–restricted T cells in all respects except one: their lack of CD4. Their appearance may be explained in one of three ways. First, the positively selecting signal that causes thymocytes to stop synthesis of either CD4 or CD8 may, with some probability, sometimes cause thymocytes to stop synthesis of both CD4 and CD8. Secondly, inhibition of synthesis of both CD4 and CD8 may actually be driven by the positively selecting signal, if the signal is high, but not quite high enough to cause the thymocyte to die. Thirdly, in TCR transgenic mice, TCRs are expressed on the surface of thymocytes before they become CD4$^+$CD8$^-$ and selection in this case may allow development of DN T cells which have never passed through the double positive stage. All of these ideas require that the selecting signals delivered to thymocytes by engagement of the DO-TCR by IA^b is at just the right level, such that additional signals from CD4 or CD8 are not required, and the signal is just large enough to allow positive selection but not clonal deletion of CD4 cells.

Several results argue in favor of the last of these hypotheses. First, DN DO-TCR$^+$ T cells appear at considerable rate in H-2^b mice, suggesting that they are not the product of some minor pathway of T cell development. Second, their CD8 genes are not as undermethylated as those of mature normal T cells which have developed through a CD8$^+$ stage. Third, these cells do not express unrelated genes under the control of the CD4 promoter, even if CD4 has never been expressed on the cells. Hence the fact that they do not express these CD4 promoter driven genes cannot be due to some selective pressure caused by CD4 itself. Finally, although DN α/β TCR$^+$ T cells are present in normal nontransgenic mice, they are a very minor population. Were their appearance to be simply a matter of the appropriate affinity of TCR for selecting ligand, one might have predicted that a number of TCRs might satisfy this requirement, and that such a population might therefore be more numerous in normal animals than it is. More likely, the appearance of α/β TCR-bearing cells of this type is dependent upon some property of the TCR transgenes themselves, in this case, their early expression on DN thymocytes.

If these DN DO-TCR T cells do arise without passing through a CD4$^+$CD8$^+$, double-positive stage, what relevance does this have for the development of T cells in normal mice? In normal animals TCR β chains are expressed on DN thymocytes, but TCR α chains are not, so there is no opportunity for positive selection of DN thymocytes in such animals. In normal animals TCR β chains are expressed on DN thymocytes bound to pre-Tα chains and associated

with CD3 (54). It is thought that this combination signals to the thymocyte and thus induces expression of the CD4 and CD8 genes as well as rearrangements at the α chain locus (55, 56). It has been suggested that this signal is induced by binding of the TCR β chain/pre-Tα complex to some unknown ligand. If this is so, clearly ligation of complete TCR α/β combinations at the DN stage delivers a different signal, since this latter ligation does not appear to induce CD4 and CD8 expression. Perhaps the difference is a matter of the affinity with which TCRβ/pre-Tα or TCR-α/β are engaged by their respective ligands.

Finally, various theories have been suggested to account for the fact that the accessory proteins, CD4 or CD8, expressed by T cells, usually match the restriction of the TCRs they bear, for class II or class I MHC. Instructive theories suggest that positive selection of thymocytes by reaction with class II dictates CD4 expression by the selected cells (17, 18, 57) whereas selective theories suggest that thymocytes make a random choice and are then selected to mature if they express the CD4 or CD8 protein which matches the restriction specificity of their TCRs (13–16). The experiments described in this article demonstrate, as many experiments have done in the past, that this instruction cannot be absolute since clearly DO-TCR–bearing T cells that have been selected on IA^b do not express CD4.

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