Distinctive Selection Mechanisms Govern the T Cell Receptor Repertoire of Peripheral CD4<sup>−</sup>CD8<sup>−</sup> α/β T Cells
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Summary

The T cell receptor (TCR) repertoire of CD4<sup>+</sup> and CD8<sup>+</sup> α/β T cells is heavily influenced by positive and negative selection events that occur during T cell development in the thymus. The coreceptors CD4 and CD8 appear to be essential for this selection to occur. To gain insight into whether T cells that express TCR α/β but lack either coreceptor (CD4<sup>−</sup>CD8<sup>−</sup> TCR α/β double-negative [DN] cells) are also subject to positive and negative selection, and whether selection can occur in the absence of coreceptors, we have performed an extensive immunogenetic analysis of the TCR Vβ repertoire of α/β DN cells in lymph nodes of normal mice. Our results show that α/β DN cells appear to be unaffected by clonal deletion of Vβ5 and Vβ11 in I-E<sup>+</sup> expressing mice, and do not undergo deletion of Vβ6- and Vβ8.1-expressing T cells in Mls-1<sup>−</sup> positive mice. They are also unaffected by positive selection of Vβ17a<sup>+</sup> T cells in the context of I-A<sup>+</sup>. The results suggest that most selection events require the participation of CD4 and CD8, while α/β DN cells are unselected. This argues that most α/β DN cells probably have never expressed CD4 or CD8. However, a unique form of repertoire selection occurs: enrichment of Vβ17a<sup>+</sup> α/β DN cells in I-E<sup>+</sup> mice. This could be an instance of coreceptor-independent selection.

Mature T cells that express TCR α and β chains (TCR α/β) usually also express either CD4 or CD8, coreceptor molecules that are thought to stabilize the antigen peptide/MHC/TCR interaction during T cell antigen recognition and to participate in transmembrane signal transduction during T cell activation (1-4). The majority of CD4<sup>+</sup> and CD8<sup>+</sup> α/β T cells develop in the thymus from CD4<sup>−</sup>CD8<sup>−</sup> (double-negative [DN])<sup>1</sup> precursors, which do not express TCR α/β on the cell surface, and pass through a CD4<sup>+</sup>CD8<sup>+</sup> (double-positive [DP]) maturation stage (5, 6). As this process occurs, the developing lymphocytes rearrange their α and β chain genes and begin to express low levels of TCR α/β on their surface (5, 6). The DP intermediates then increase surface level of TCR α/β expression, turn off either CD4 or CD8 expression, and progress to functionally distinct CD4<sup>+</sup>CD8<sup>−</sup> or CD4<sup>+</sup>CD8<sup>−</sup> mature T cells (5, 7).

It is abundantly clear that the repertoire of CD4<sup>+</sup> and CD8<sup>+</sup> α/β T cells is shaped by selection events during their development in the thymus. As a way to maintain self-tolerance, T cells with self-reactive TCR α/β are physically deleted in the thymus (negative selection) (8-15). To ensure that the T cells produced are functionally competent and immunologically useful to the host, only T cells bearing TCR α/β capable of recognizing foreign antigen in the context of self-MHC molecules are given signals that lead to full maturation (positive selection) (14-18).

Both negative and positive selection have been directly documented by the influence of superantigen and/or MHC molecules on the usage of products of the VB gene families. It has been shown that T cell recognition of superantigens and/or MHC molecules is dominated by TCR VB segments, expressed with any TCR α chain (9-13). T cells expressing Vβ17a (Vβ17a<sup>+</sup> T cells) are reactive to the class II MHC molecule I-E, plus an endogenous superantigen, and are thus negatively selected in I-E-expressing mouse strains (9). T cells that express Vβ8.1 or Vβ6 (Vβ8.1<sup>+</sup> or Vβ6<sup>+</sup> T cells), which impart TCR reactivity to Mls-1<sup>+</sup>, an endogenous superantigen encoded by the retrovirus Mtv-7, plus most class II alleles, are eliminated intrathymically in Mls-1<sup>+</sup> mice (10-12, 19). In addition to Vβ17a<sup>+</sup> T cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing Vβ5 or Vβ11 are also deleted in most I-E<sup>+</sup> strains of mice because of their reactivity to I-E plus superantigens encoded by endogenous retrovirus Mtv<sup>−</sup>, Mtv<sup>−</sup>9, or Mtv<sup>−</sup>11 (9, 13, 19). Positive selection has been exemplified by the selective effect of MHC haplotype on the

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<sup>1</sup>Abbreviations used in this paper: DN, double negative; DP, double positive.
expression of Vβ17a (13, 14, 20). The CD4+ T cells expressing Vβ17a, in the absence of I-E, are selectively increased in H-2d mice (13, 14). It is believed that both positive and negative selection events involve the coreceptors CD4 and CD8 (5). During T cell ontogeny in the thymus, the selection processes act on CD4+CD8+ cortical thymocytes (5, 8, 15, 16, 21), and antibodies against either the coreceptor or its MHC ligand specifically block the T cell maturation into either CD4+ or CD8+ SP cells (22-24).

Recently, a minor subpopulation of TCR α/β+ T cells that expresses neither CD4 nor CD8 (α/β DN cells) has been identified in both the thymus and the periphery of normal animals (25-30). In the murine thymus, α/β DN cells express an unusual TCR repertoire with disproportionately high expression of Vβ8.2 (25-28). In the periphery, the α/β DN cells exist in the lymph nodes, bone marrow, peripheral blood, and skin of normal animals (31-33). Although T cells with this phenotype are normally very rare, they are more abundant in the peripheral lymphoid tissues of ipr and gid mutant mice (34, 35) and some transgenic mice (8, 36, 37). The developmental origin and functions of α/β DN cells are entirely unknown.

To examine whether α/β T cells without coreceptors are also subject to positive and negative selection, and whether selection can occur in the absence of receptors, we have conducted an extensive immunogenetic analysis of the Vβ repertoire of α/β DN lymph node cells. The results indicate that most α/β DN cells are unselected, suggesting that most selection events require the participation of the CD4 and CD8 coreceptor molecules. However, an unexpected genetic effect on Vβ expression was observed: enrichment of Vβ5/6α/β DN cells in I-E+ mice. This could be an instance of coreceptor-independent selection.

Materials and Methods

Mice. CBA/J, CBA/Ca, SWR/J, C57L/J, and C57BR/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hybrid (SWR x C57L)F1, and (SWR x C57BR)F1 mice were bred in the Immunobiology Mouse Unit at Yale University School of Medicine. All mice were kept in a pathogen-free environment and used at 4-8 wk of age.

Antibodies. The following anti-TCR antibodies were used as supernatants of hybridoma cultures: H57.597-2.1, anti-Cβ (17); RR4-7, anti-Vβ6 (10); RR3-15, anti-Vβ11 (38); MR.9-4, anti-Vβ5 (39); F23.1, anti-Vβ8.1 8.2-8.3 (40); KJ16-156, anti-Vβ8.1 8.2 (41); F23.2, anti-Vβ8.2 (11); KJ23, anti-Vβ17a (9); and GL3-3A, anti-γδ (42). GK1.5-FITC (anti-CD4) (43) and 3.168-FITC (anti-CD8) (44) used in immunofluorescence staining were made and tested in our laboratory. Either goat anti-mouse Ig antisera (batch G48, generously provided by Dr. Michael Parkhouse, National Institute for Medical Research, London, UK) or goat anti-mouse IgG (Sigma Immuno Chemicals, St. Louis, MO) were used in panning to deplete B cells.

Cell Preparation. Single-cell suspensions were prepared from the pooled peripheral (inguinal, axillary, cervical, popliteal, and mesenteric) lymph nodes of individual mice. After washing twice with PBS containing 1% BSA, B cells were depleted by panning as described (45). Briefly, petri dishes were incubated with either purified G48 (1 mg/ml) or anti-mouse Ig (5 μg/ml) for 30 min at room temperature, then unoccupied protein-binding sites on the dishes were blocked by incubating with PBS-1% BSA for 20 min at room temperature. Single-cell suspensions were then loaded onto the petri dishes at 4 x 10⁶ cells/dish. After incubation for 45 min at room temperature, nonadherent cells were harvested by swirling the plates and gently aspirating the cell suspension. Hand-picked cells were then washed twice and checked for purity by staining with anti-mouse Ig-PE (Biomeda, Foster City, CA) and FACS® analysis (Becton Dickinson & Co., Mountain View, CA). B cell contamination was always <0.5%.

Immunofluorescence Staining and FACS® Analysis. 106 B cell-depleted LN cells were suspended in 50 μl PBS-1% BSA. For studying the Vβ expression of α/β DN cells, 50 μl (1:1) of culture supernatants of various anti-TCR mAbs was added. After incubation for 30 min on ice, cells were washed once in PBS-1% BSA and resuspended in 50 μl staining buffer containing 50 μg/ml PE anti–Rat Ig (Biomedica), incubated for 30 min on ice, and washed as above. Cells were then suspended and incubated on ice for 30 min in 50 μl PBS-1% BSA containing 100 μg/ml rat IgG to block the free binding sites on the second layer. Cells were finally stained with a mixture of GK1.5-FITC and 3.168-FITC. Stained cells were fixed in 1% paraformaldehyde (PFA) and analyzed using FACSScan® flow cytometer and software (Becton Dickinson & Co.). Dead cells were excluded by forward and side scatter gating. For studying the Vβ expression of α/β DN, a live gate was set on the CD4+CD8+ population. Analysis was performed on at least 2,000 events. The data shown are the percentage of cells positive for a particular Vβ, divided by the percentage positive for CB.

Statistical Analysis. Statistical significance of differences was calculated using the Mann-Whitney U test.

Results

Identification of Peripheral α/β DN T Cells by FACS® Analysis of Unfractionated LN T Cells. The presence of α/β DN T cells in the periphery has been reported as a minor subpopulation (31). Since the subtypes of the phenotype of α/β DN cells and their behavior on a nylon wool column could not be foreseen, we started our study with purified LN T cells prepared by depleting B cells, using panning on anti-Ig-coated petri dishes. We were unwilling to use depleting antibodies apart from anti–mouse Ig, because of the risk that selective cell loss would destroy the integrity of the α/β DN repertoire. The α/β DN were identified by two-color FACS® analysis of the B-depleted, but otherwise unfractionated, LN T cells. As is shown in Fig. 1, the staining pattern was such that the CD4+, CD8+, and CD4-CD8- subpopulations were readily distinguishable. Like the γ/δ T cells, the α/β DN LN cell subpopulation was distinct from the CD4+ and CD8+ T cells. The α/β DN subpopulation was consistently detectable in the lymph nodes of all the mouse strains that we studied. It accounted for 0.2-0.8% of the B cell-depleted LN cells. A population of γ/δ cells, consisting of 0.6-1.2% of the B cell-depleted LN cells, made up the majority of the CD4-CD8- LN cells. The proportion of α/β DN among T cells in the spleen was similar to that in the lymph nodes. The α/β DN LN T cells were also distinct from CD4+ and CD8+ T cells in their surface levels of TCR α/β expression. The TCR density of the α/β DN cells was somewhat lower than that of the CD4+ and CD8+ T cells (Figs. 1 and 2).
Figure 1. Two-color staining of B cell-depleted LN cells for CD4+CD8− α/β and γ/δ T cells. LN cells were depleted of B cells by panning as described in Materials and Methods. Native H57.597-2.1 (anti-pan-TCR−/−B) or GL3-3A (anti-pan-TCR−/−8) antibodies were used as the first layer and PE anti-rat Ig as the second layer in red fluorescence, vs. a mixture of GK1.5-FITC (anti-CD4) and 3.168-FITC (anti-CD8) in green fluorescence. Both CD4+CD8−α/β and γ/δ T cells are readily detectable as distinct subpopulations. The contour figures were plotted on logx0 scale with 250,000 events.

These preliminary studies not only confirmed the existence of α/β DN cells as a minor but distinct subpopulation in the lymph nodes of normal mice, but also demonstrated the feasibility of using this protocol to study the expression of various VBs in α/β DN CD4+ and CD8+ T cells by setting electronic gates on the three distinct subpopulations (Fig. 3). This protocol was therefore utilized in the following studies. We used this approach rather than purifying α/β DN cells by cytotoxic elimination of CD4+ and CD8+ cells because we and other investigators (31) had found that it is difficult to obtain pure peripheral CD4+CD8− T cells by complement depletion using anti-CD4 and anti-CD8 mAbs. Staining with anti-rat Ig-FITC would routinely reveal up to 40–60% contamination of CD4low and CD8low cells that usually failed to be stained with anti-CD4 and anti-CD8 direct conjugates due to steric blocking. The difficulties in obtaining highly pure α/β DN cells by complement depletion may explain the disparity between our results and the results reported by other investigators (46).

The TCR Repertoire of Peripheral α/β DN Cells Is Not Influenced by I-Aq-induced Positive Selection. It has been demonstrated that the expression of the Vβ17a gene segment seems to be under the control of positive selection by I-Aq, since Vβ17a+ T cells are selectively increased in the CD4+ T cells in mice that express I-Aq, such as SWR (H-2q) (13, 14). To study whether the repertoire of α/β DN T cells was also influenced by similar positive selection, we analyzed and compared the Vβ17a usage by α/β DN LN T cells of SWR/J (H-2q), C57L/J (H-2b), and (SWR × C57L)F1 (H-2q × b) mice. The data are summarized in Fig. 4. In agreement with the literature, we have shown that the Vβ17a expression was significantly increased in CD4+ LN T cells in SWR/J compared with that in C57L/J mice, with CD4+ cells of (SWR × C57L)F1 (H-2q × b) mice having intermediate levels. This suggests that H-2q haplotype influences Vβ17a usage in α/β DN T cells.
Influenced by Mls-1\(^{-}\)-induced Negative Selection. Since most samples were
4. This was not a sampling artifact of the small number of
cells falling inside the live gate, since staining with F23.1 for
V\(\beta\)8, which is deleted from the genome of SWR/J and
C57L/J mice, consistently gave no significant staining above
the background (0.3 ± 1.9%).

The TCR Repertoire of Peripheral \(\alpha/\beta\) DN Cells Is Not
Influenced by Mls-1\(^{-}\)-induced Negative Selection. T cells bearing
TCRs that are potentially self-reactive are physically deleted
at the DP stage during their development in the thymus (9-15).
It has been well documented that V\(\beta\)6\(^{+}\) and V\(\beta\)8.1\(^{+}\) T cells
are reactive to Mls-1\(^{-}\), an endogenous superantigen encoded
by the retrovirus Mtv-7 (10-12, 47). The V\(\beta\)6\(^{+}\) and V\(\beta\)8\(^{+}\)
T cells are, therefore, deleted intrathymically in Mls-1\(^{-}\) strains
of mice (10-12). To examine the possible effect of negative
selection by Mls-1\(^{-}\) superantigen on the repertoire of peripheral
\(\alpha/\beta\) DN cells, we studied the usage of a variety of V\(\beta\) segments in CBA/J (H-2\(^{k}\), Mls-1\(^{+}\)) vs. CBA/Ca (H-2\(^{k}\),
Mls-1\(^{+}\)) mice. In contrast to the selected repertoire of CD4\(^{+}\)
and CD8\(^{+}\) cells (data not shown), which was consistent
with the literature, the repertoire of \(\alpha/\beta\) DN LN T cells
appeared not to be influenced by the presence of Mls-1\(^{-}\) su-
perantigen. The \(\alpha/\beta\) DN LN T cells of CBA/J mice expressed
a similar level of V\(\beta\)6 and V\(\beta\)8.1 expression to those
of CBA/Ca mice (Fig. 5) (\(p > 0.1\) for all differences). In ad-
dition, both CBA/Ca and CBA/J mice are H-2\(^{k}\), but contained a significant, although quite variable,
percentage of \(\alpha/\beta\) DN LN T cells expressing V\(\beta\)81 or V\(\beta\)85.
In some extreme cases, ∼20-30% of orb DN expressed V\(\beta\)311
in I-E\(^{+}\) mouse strains (6, 25, 26), overexpression of V\(\beta\)88.2 (>30% of \(\alpha/\beta\) DN) of \(\alpha/\beta\) LN DN T cells was only observed in 3 of 13 mice (data not shown).

Peripheral \(\alpha/\beta\) DN Cells Expressing V\(\beta\)17a Are Enriched in
I-E\(^{+}\) Mouse Strains. The V\(\beta\)17a\(^{+}\) T cells have been found
to reactive to MHC I-E molecules plus a superantigen (9),
and the expression of V\(\beta\)17a by CD4\(^{+}\) and CD8\(^{+}\) cells is
therefore significantly diminished in I-E\(^{+}\) mouse strains (9).
After finding that the usage of V\(\beta\)6 and V\(\beta\)8.1 of \(\alpha/\beta\) DN
LN T cells appeared to be un influenced by Mls-1\(^{+}\) superan-
tigen, we asked whether it was also true for the usage of
V\(\beta\)17a in the presence of I-E. To study this, we analyzed and
compared the V\(\beta\)17a expression by \(\alpha/\beta\) DN LN T cells from
SWR/J(I-E\(^{-}\)), C57BR/J(I-E\(^{+}\)), and (SWR \(\times\) C57BR)\(^{F1}\) mice.
Based upon what had been found in the Mls-1\(^{+}\) system, we predicted that the usage of V\(\beta\)17a by \(\alpha/\beta\) DN
LN T cells would be similar in C57BR/J, SWR/J, and (SWR
\(\times\) C57BR)\(^{F1}\) strains (Fig. 7) (\(p < 0.05\) and \(p < 0.025\), respec-
tively). In some cases, over half of the \(\alpha/\beta\) DN LN T cells
in C57BR/J and (SWR \(\times\) C57BR)\(^{F1}\) mice expressed
C57BR/J (I-E +), and (SWR x C57BR)F1 mice were depleted of B cells, as previously discussed, since staining for V~8 cells. Background staining has been subtracted.

Figure 7. Enrichment of Vβ17a + α/β DN LN T cells in C57BR/J was similar to the background staining (0.3 ± 2.8%).

C57BR)F1 strains (Fig. 7). This variation was unlikely to be from technical, as previously discussed, since staining for Vβ8 was similar to the background staining (0.3 ± 2.8%).

Discussion

The repertoire of peripheral α/β DN cells appears quite different from that of CD4+ and CD8+ cells. Two main findings are described in this report.

First, there was no evidence for selection of the Vß repertoire of most α/β DN cells. We looked for negative selection of Vß5+ and Vß11+ α/β DN cells in I-E+ mice and clonal deletion of Vß6+ and Vß8.1+ α/β DN cells by Mls-1a superantigen; in neither case was deletion observed. We also sought evidence for positive selection of Vß17a+ cells in H-2k mice; there was none. Thymic selection of the CD4+ and CD8+ T cell repertoire has been shown to occur at the DP stage of T cell differentiation (5, 8, 15, 21), and has in several systems been shown to depend on the involvement of CD4 molecules (24, 46). Our finding of an unselected repertoire of α/β DN cells is hard to reconcile with the passage of these cells through a selectable CD4+CD8+ stage, and suggests that they arise as DN and have never expressed CD4 or CD8 molecules. Our data are consistent with the findings in transgenic mice that CD4+CD8- cells that express the transgene TCR appear not to be subject to selection by the MHC molecules on thymic epithelium (49, 50). If these cells are a T cell lineage that does not require the participation of CD4 or CD8 to complete differentiation, and therefore are not subject to thymic selection, how are they related to the main stream of T cell differentiation taking place in the thymus? Guidos et al. (31) have shown that donor-derived α/β DN cells could readily be detected in the periphery after intrathymic reconstitution with CD4+-CD8- precursor thymocytes, suggesting that cells that rearrange both α and β chains of the TCR in the thymus without CD4 or CD8 expression could be exported to the periphery. Rearrangement of the TCR β genes appears to be complete before the cells leave the DN stage. It is possible that α/β DN cells arise when the TCR α locus also completes rearrangement before the CD4 and CD8 genes are expressed. Cells could then be exported from the thymus, and removed from the environment in which induction of CD4 and CD8 expression occurs. The observation that α/β DN cells are greatly increased in the periphery of TCR α and TCR α/β transgenic mice (8, 36, 37), where the TCR α chain is expressed earlier in development, supports this view. The export of such cells also argues that export from the thymus may require prior TCR expression, but not positive selection. Although the repertoire of thymic and peripheral α/β DN supports this model, there is some contrary evidence. The demethylation of the CD8α gene in thymic α/β DN cells has been interpreted as evidence of prior CD8 expression (27, 51). The CD8α gene is methylated before its expression but is demethylated once it has been expressed (52, 53). The CD8α gene in CD4+ cells is demethylated, which is consistent with the derivation of CD4+ SP cells from DP thymocytes (53).

Alternatively, these cells could arise by an yet undefined extrathymic pathway. An increased frequency of α/β DN cells has been reported in nude mice (54), and observed in ATx BM mice (L. Huang, unpublished observation). Von Boehmer et al. (49) have shown that the DN cells expressing the transgenic TCR in transgenic mice are thymus dependent. It remains to be elucidated, however, whether DN cells expressing the transgene TCR α/β, which are much more abundantly present in transgenic mice, are representative of the minor population of α/β DN cells in normal unmanipulated mice. The presence of rearranged TCR genes in the transgenic mice may allow the expansion of minor lineage pathways. At present, we have no conclusive evidence to distinguish between these two models. A systematic study of the phenotype and repertoire of α/β DN cells in nude and ATx BM mice is under way, and we hope this will shed light on the problem.

An unexpected finding described in this report is that the frequency of Vß17a+ α/β DN cells is increased in I-E+ mice. This is a striking inversion of the negative selection of Vß17a+CD4+ and CD8+ cells seen in the same mice. Two possibilities exist to account for this unique selection pattern. Either these cells, unlike other α/β DN cells, arose as a result of downregulation of a previously expressed CD4 or CD8 coreceptor, or this particular form of selection of the Vß repertoire can occur in the absence of coreceptors. If the Vß17a+ α/β DN in I-E+ mice arose as a result of coreceptor downregulation, this could be a mechanism by which cells with an affinity for I-E too low to result in clonal
deletion in the thymus are rendered tolerant in the periphery. There are precedents for this in the behavior of H-Y-specific transgenic T cells transferred into male nude hosts, where H-Y-specific CD8+ T cells that were not eliminated downregulated their surface expression of CD8 as well as TCR (55). If these Vß17a+ cells are indeed cells that avoided clonal deletion, they might have an unusually low affinity for I-E because of a limited set of TCR α or β segments, which compromise the interaction between Vß17a and I-E.

Alternatively, the high frequency of Vß17a+ α/β DN cells could have resulted from positive selection or peripheral expansion without the involvement of a coreceptor. Coreceptor independence has been proposed to be a property of T cells whose TCR has as unusually high affinity for their antigen-MHC ligand (56). In terms of this model, we would postulate that the affinity between Vß17a and I-E is higher than that of the other selective interactions.

The two models make different predictions about the affinity of TCR for I-E in the Vß17a+ α/β DN cells in C57BR/J mice. We are currently testing these predictions by preparing a panel of hybridomas from c~//3 DN of C57BR. whose TCK has as unusually high affinity for their antigen-cells could have resulted from positive selection or peripheral regulation their surface expression of CD8 as well as TCK H-Y-specific CD8+ T cells that were not eliminated down-transgenic T cells transferred into male nude hosts, where There are precedents for this in the behavior of H-Y-specific deletion in the thymus are rendered tolerant in the periphery.

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