Allelic Exclusion of the TCR α-Chain Is an Active Process Requiring TCR-Mediated Signaling and c-Cbl

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Allelic Exclusion of the TCR α-Chain Is an Active Process要求

Requiring TCR-Mediated Signaling and c-Cbl

Nathalie Niederberger,* Kaisa Holmberg,* S. Munir Alam,2* Wayne Sakati,* Mayumi Naramura,3† Hua Gu,† and Nicholas R. J. Gascoigne4*  

Phenotypic allelic exclusion at the TCRα locus is developmentally regulated in thymocytes. Many immature thymocytes express two cell surface α-chain species. Following positive selection, the vast majority of mature thymocytes and peripheral T cells display a single cell surface α-chain. A posttranslational mechanism occurring at the same time as positive selection and TCR up-regulation leads to this phenotypic allelic exclusion. Different models have been proposed to explain the posttranslational regulation of the α-chain allelic exclusion. In this study, we report that allelic exclusion is not regulated by competition between distinct α-chains for a single β-chain, as proposed by the dueling α-chain model, nor by limiting CD3 ζ-chain in mature TCRhigh thymocytes. Our data instead favor the selective retention model where the positive selection signal through the TCR leads to phenotypic allelic exclusion by specifically maintaining cell surface expression of the selected α-chain while the nonselected α-chain is internalized. The use of inhibitors specific for Lck and/or other Src kinases indicates a role for these protein tyrosine kinases in the signaling events leading to the down-regulation of the nonselectable α-chain. Loss of the ubiquitin ligase/TCR signaling adapter molecule c-Cbl, which is important in TCR down-modulation and is a negative regulator of T cell signaling, leads to increased dual α-chain expression on the cell surface of double-positive thymocytes. Thus, not only is there an important role for TCR signaling in causing α-chain allelic exclusion, but differential ubiquitination by c-Cbl may be an important factor in causing only the nonselected α-chain to be down-modulated. The Journal of Immunology, 2003, 170: 4557–4563.

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ifferent forms of allelic exclusion regulate expression of the two chains of the TCR, α and β (1, 2). Generally, the presence of a productively rearranged TCRβ gene impedes further β-chain gene rearrangement on the other locus (3). This is not the case for the TCRα gene (1, 2). During the CD4+CD8+ (double-positive (DP)) phase of thymocyte development, both alleles of the α-chain gene rearrange until a positively selectable heterodimer is formed with the previously rearranged β-chain (4). When positive selection occurs, recombination-activating genes 1 and 2 are turned off, stopping further rearrangement (5, 6). A posttranslational mechanism ensures that only one α-chain will be present on the cell surface of mature T cells (1, 2, 7, 8). Such a mechanism has also been shown to operate in double αβ TCR-transgenic mice (9). Therefore, most mature thymocytes and peripheral T cells show phenotypic allelic exclusion and display only one cell surface α-chain, whereas the immature TCRlow DP cells frequently express two cell surface α-chains (8, 10, 11).

Different models for phenotypic allelic exclusion of the α-chain have been proposed. The dueling α-chain model invokes competition between two α-chain proteins for a limiting supply of β-chain protein, when the TCR level on the cell surface increases during positive selection (2, 8, 10). A variant of this model is that the limiting factor is not the β-chain, but the CD3 ζ-chain, a component of the TCR-CD3 complex. The availability of CD3ζ generally limits the level of TCR on the cell surface (12). The ζ-chain might play an important role in phenotypic allelic exclusion of α-chain by stabilizing preformed αβ pairs and thus preferentially stabilizing the αβ combination with the highest interchain affinity available to the cell (2). Finally, the selective retention model proposes that the positively selected αβ combination is maintained on the cell surface while the nonselected α-chain is down-modulated or retained within the cell (11). Recent evidence indicates that TCR is constitutively endocytosed and recycled to the cell surface, so that down-modulation may instead be a lack of recycling to the cell surface (13).

Stimulation through the TCR initiates a cascade of signaling events, including the phosphorylation of CD3 components by the CD4/CD8 coreceptor-associated protein tyrosine kinase Lck. The overexpression of Lck in developing thymocytes impedes β-chain gene rearrangement during thymocyte development (14), whereas an inactive form of Lck blocks allelic exclusion of the β-chain (15). Expression of a constitutively active form of Lck in T cells induces internalization and lysosomal degradation of TCR (16). Thus, different levels of Lck activity generated by TCR signaling could affect allelic exclusion of α-chain through signals leading to TCR down-regulation during positive selection.

The adapter protein c-Cbl is involved in the regulation of TCR-mediated signaling and is phosphorylated after TCR stimulation (17, 18). c-Cbl reduces T cell activation through binding to, and

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5Abbreviations used in this paper: DP, double positive; PP1, phosphatase 1.
increasing degradation of, $\zeta$-associated protein-70 and Fyn (18–20). c-Cbl knockout mice show increased T cell signaling (21, 22). Most strikingly, c-Cbl has recently been demonstrated to function as a ubiquitin ligase (23, 24). Ubiquitination of cell surface receptors is an endocytotic signal that can target them for degradation in proteosomes or by lysosomes (25). This suggested that c-Cbl could be involved in endocytosis and/or degradation of TCR after activation, because ubiquitination of TCR occurs after TCR-mediated stimulation and requires Lck activity (26, 27). Recent data confirm that c-Cbl and its close relative Cbl-b are closely involved in the down-modulation and degradation of TCR after activation (28).

DP thymocytes normally have a TCRlow phenotype, but those of c-Cbl knockout mice show up-regulation of TCR (21, 22), similar to that seen with inhibition of Lck activity in fetal thymocytes (16), suggesting that c-Cbl has a role in maintaining the low level of TCR seen in DP thymocytes. Thus, c-Cbl appears to be intimately involved in negative regulation of TCR signaling and in TCR internalization and degradation. This suggests that ubiquitination could be a method for marking stimulated TCRs to distinguish them from the unstimulated, making ubiquitination a candidate for regulation of $\alpha$-chain allelic exclusion. Transient protection of TCR from degradation has been noticed after stimulation (29), and stimulated and unstimulated TCR may be internalized by ubiquitination (30), suggesting that c-Cbl has a role in maintaining the low level of TCR seen in DP thymocytes. Furthermore, TCR endocytosis and recycling are features of resting T cells, with signaling through the TCR causing intracellular retention and degradation of TCR (13).

In this study, we compare different models of phenotypic allelic exclusion of the TCR $\alpha$-chain. Our results favor the selective re-

### Materials and Methods

**Mice**

C57BL/6 (B6) mice were bred and maintained at The Scripps Research Institute. The $\zeta$-deficient mice with transgenic $\zeta$-chain expressed at 2-fold higher than normal level (CD$\zeta^{+/+}$) were obtained from Dr. P. Love (National Institutes of Health). The $\alpha$-chain knockout mice (Tcra$^{-/-}$; backcrossed to B6 for seven generations) and beige mutant mice (B6$^{bg/bg}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). Tcra hemizygous (or $\alpha^{+/+}$) animals were produced by breeding (or $\alpha^{+/+}$) with B6 mice. The c-Cbl knockout mice have been described previously (21). OT-I TAP$^{+}$ mice (The Jackson Laboratory, Bar Harbor, ME) were obtained from Dr. L. Berg (University of Massachusetts, Worcester, MA). All mice were maintained at The Scripps Research Institute, and all experiments were performed in accord with the guidelines of the Animal Care and Use Committee of The Scripps Research Institute.

**Flow cytometry and Abs**

Single-cell suspensions from thymi of 4- to 8-wk-old mice or from cultured fetal day 17 B6 thymi were prepared. For each staining, 2.5 × 10$^5$ thymocytes were resuspended in 50 $\mu$L of PBS containing 1% BSA and 0.1% sodium azide (flow buffer (FB)) and appropriate Abs. Unless otherwise noted, the Abs used in this study were purchased from BD Pharmingen (San Diego, CA). The staining was done in the dark for 30 min at 4°C. Cells were washed, resuspended in FB, and analyzed with a FACSCalibur instrument (BD Biosciences, Mountain View, CA) using CellQuest software. All the Abs used for staining were directly conjugated to PE, FITC, allophycocyanin, or PerCP. They were anti-Var2, -Var3.2, -Var8, -Var11, -Var8, -CD4, -CD8, and -TCR$\beta$. Control staining was performed with irrelevant rat IgG2b. The Abs used for the TCR down-modulation experiment were goat anti-rat, anti-mouse (purchased from Sigma-Aldrich, Irving, CA), and anti-hamster Ab as well as purified anti-Var2 (clone B20.1), rat IgG3; obtained from B. Malissen (Centre National de la Recherche Scientifique, Marseille-Luminy, France), anti-Var2.1,5,2 (clone MR6-4; mouse IgG2b; obtained from O. Kanagawa (Washington University, St. Louis, MO)), and anti-CB (clone H57-597; mouse IgG; obtained from R. Kubo (Cytel, San Diego, CA)) mAbs.

**Fetal thymic organ culture**

Thymi from day 17 fetuses of B6 mice were cultured as described previously (16). Briefly, thymi were cultured overnight in organ culture medium in the presence of 20 $\mu$L of the pyrazolopyrimidine PPI (Axoxra, San Diego, CA) dissolved in DMSO or the DMSO vehicle alone. Organ culture medium was prepared by mixing RPMI 1640 and DMEM (1:1) media supplemented with 2 mM glutamine, 100X MEM nonessential amino acids, 20 mM HEPES, 1 mM sodium pyruvate, and 50 $\mu$L 2-ME. The medium was filtered using Millipore (Bedford, MA) 0.22 $\mu$m filtration system. Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN) 1% was added. Gel foam sterile sponges were prewetted in 3 ml of medium in 6-well plates. Millipore filters were added on the top of the sponges. Fetal day 17 thymi were placed on top of the filter and cultured overnight at 37°C/5% CO2. The thymi were prepared for flow cytometry analysis.

**Adult thymocyte culture and Ab-mediated TCR down-modulation**

Single-cell suspensions of thymocytes from 4- to 8-wk-old B6 or hemizygous Tcra$^{+/+}$ mice thymi were prepared. Thymocytes (4 × 10$^5$ cells/ml) were resuspended in 6 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100X penicillin G sodium (10,000 U/ml)/10,000 $\mu$g/ml streptomycin sulfate, and 0.05 mM 2-ME. Cells were cultured overnight at 37°C/5% CO2 in 6-well tissue culture plates. For the inhibitor assays, 10$^4$ U/ml bovine serum albumin (BSA) and 20 $\mu$L PPI (or DMSO vehicle) were added (these concentrations were determined from titrations as having specific activity without significant toxicity). Following the incubation, thymocytes were fixed and analyzed for TCR down-modulation experiments, goat anti-TCR, anti-hamster, or anti-mouse Ab (10 $\mu$L/ml) were immobilized on 96-well round-bottom tissue culture plates overnight at 4°C. Plates were washed with PBS and further coated with 2 $\mu$L/ml of purified anti-Var2, anti-Var2.1,5,2, or anti-CB mAbs for 4 h at 37°C. Thymocytes prepared as above were cultured (5 × 10$^4$/well) for 18 h at 37°C/5% CO2.

**Capping of TCR**

Single-cell suspensions of thymocytes from 4- to 8-wk-old OT-I (TAP$^{+/-}$) mice were prepared. Thymocytes (3 × 10$^5$ cells/staining) were resuspended in 100 $\mu$L of PBS containing 1% BSA and 0.1% sodium azide (FB). To induce capping, cells were incubated with biotinylated anti-Var2 or anti-Var2.5 for 30 min at 4°C, washed, resuspended in FB, and stained with streptavidin-FITC for 10 min on ice. Thymocytes were then incubated at 37°C, and cap formation was observed using a DeltaVision deconvolution microscope system (Applied Precision, Issaquah, WA) as described (33).

**Biacore analysis**

Comparison of affinities of Var2 and Var5 to OT-I TCR was performed on a Biacore 2000 instrument (Biacore, Piscataway, NJ). Five hundred to 600 resonance U of biotinylated mAb (anti-Var11, -Var2, -Var5, and -CB) were immobilized on an SA-sensorchip (Biacore). The surface was allowed to stabilize for 30 min at 10 $\mu$L/min with running buffer (PBS and 0.005% surfactant P20). Soluble TCR was produced in insect cells, as described earlier, using a baculovirus vector (34, 35). TCR was purified with nickel-nitritotriacetic acid (Qiagen, Valencia, CA) and further by size exclusion on a Superdex HR200 column (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove aggregated proteins. For injections, TCR was diluted in the running buffer to from 70 nM to 8 $\mu$L. Binding of OT-I TCR to different mAbs was monitored with 3.5-min injection and 400-s dissociation at 25°C with a flow rate of 15 $\mu$L/min. The surface was regenerated with 10-$\mu$L injection of 10 mM glycine (pH 2.0) at 25 $\mu$L/min. Before analysis, nonspecific binding to anti-Var11 was subtracted, and instrument noise was removed by subtracting a buffer injection from all curves. Analysis of kinetic rate constants was performed with BIAevaluation 3.1 software (Biacore) using global fitting with a simple Langmuir 1:1 binding model.
Results

Phenotypic allelic exclusion is not the result of a competition between two α-chains for a single β-chain

The α-chain competition or dueling α-chain model for phenotypic allelic exclusion suggests that two α-chain proteins compete for binding to the single β-chain species available (2, 8, 10). The competition is hypothesized to start as the β-chain becomes limiting due to increased expression of α-chain during positive selection. This is the point at which the TCR level on the cell surface increases (36, 37). To test this model, we artificially up-regulated TCR expression in fetal thymocytes by blocking the activity of Lck, and other Src-family protein tyrosine kinases, with the inhibitor PP1 (38). This treatment has been shown to increase TCR expression from the previously low level of expression (16). PP1 or the control vehicle DMSO was added overnight to organ cultures of thymi from day 17 B6 fetuses. The thymi were harvested and prepared for flow cytometry analysis, gating for DP thymocytes. The level of TCR expression was measured with anti-CD3 mAb, and the proportion of cells expressing dual Vα elements was determined. The thymocytes cultured with PP1 up-regulated their TCR level as expected (16) (Fig. 1A). Furthermore, an increase in the proportion of cells expressing two α-chains on the cell surface of gated DP thymocytes was observed in the PP1-treated thymocytes (Fig. 1B). Clearly, there was no increase in competition between the α-chains when the TCR level was increased. This result is in contradiction to the dueling α-chain model, but is consistent with the selective retention model (2, 11).

We previously proposed that the β-chain expression level may be limiting in post-positive selection thymocytes, so that the competition between two α-chains for binding to β would result in phenotypic allelic exclusion of cell surface TCR (8, 10). Therefore, we used a β-chain transgenic mouse strain that has approximately eight copies of the transgene with the natural Vβ3 promoter (32), to look at thymocytes with increased levels of β-chain. Single-cell suspensions of thymocytes from the 2B4 β-chain transgenic mouse and control mice were prepared and analyzed by FACS staining. We found that, contrary to the prediction of this model, the transgenic cells had a slightly lower, rather than a higher, level of dual Vα-expressing Vβ3low cells compared with that of the nontransgenic thymocytes (data not shown).

TCR γ-chain expression level does not control TCR α-chain allelic exclusion

To determine whether CD3ζ is the factor limiting the expression of dual α-chains on the cell surface of single-positive cells, we used a CD3ζ knockout/transgenic mouse strain (CD3ζ−/−;Ctβ) (39). This is a CD3ζ−/− animal with wild-type transgenic CD3ζ chain expressed under the control of human CD2 promoter. The mice express ~2-fold higher than normal CD3ζ levels, but thymocyte development is similar to that of normal B6 mice (39). Single-cell suspensions of thymocytes from CD3ζ−/−;Ctβ and control B6 mice were prepared and analyzed by FACS staining. As expected from its important function of promoting TCR surface expression, increasing the level of expression of the protein leads to increased TCR cell surface expression (Fig. 2A). Staining of cell surface TCRs with the different anti-Vα Abs available showed a similar percentage of immature thymocytes expressing dual Vα chains on the cell surface in both strains (Fig. 2, B and C). This shows that, like the β-chain, the CD3ζ expression level does not limit the expression of dual α-chains in mature thymocytes. The CD3ζ chain facilitates the expression of TCR on the cell surface of thymocytes but does not participate in allelic exclusion of the α-chain. This figure also demonstrates that the increase in dual

FIGURE 1. Up-regulation of TCR expression in fetal thymocytes does not lead to α-chain competition and increased allelic exclusion, but the opposite. Thymi from day 17 fetal B6 mice were cultured for 16 h in the presence or absence of the Lck inhibitor PP1. Following culture, single-cell suspensions of thymocytes were prepared, and cells were analyzed for expression of the indicated TCR Vα regions. Control staining was performed with irrelevant rat IgG. A, TCR expression using the anti-Cβ reagent H57-597 for PP1-treated cells (dark line) and untreated cells (dotted line). B, Vα expression in the TCR-bearing cells from untreated (left) or PP1-treated (right) thymi. A representative experiment of three is shown. The numbers in the upper right quadrants represent the mean percentage of the Vα2− cells that were positive for the indicated second Vα chain from the three experiments. The fluorochrome on the x-axis is FITC and on the y-axis is PE. The p values from the two-tailed paired t test are as follows: IgG, p = 0.4 (not significant); Vα11, p = 0.015; Vα3.2, p = 0.042; and Vα8, p = 0.010.
show increases in dual Vα/β Tcra cytes from B6 mice and mice hemizygous for Tcra (Fig. 1). This is consistent with selective retention, but not increased without the induction of positive selection or allelic exclusion (31). We cultured the thymocytes with anti-Vα2, anti-Cβ, or anti-Vβ5, with or without the inhibitors herbimycin A (inhibitor of the Src family kinases) (38). Anti-Vβ5 effectively modulated expression of all TCR components measured (Cβ, Vβ5, and Vα2) (Fig. 3A and data not shown), whereas anti-Vα2 did not cause down-modulation of Vα2 or TCR in general (Fig. 3B and data not shown). Anti-Cβ gave similar down-modulation to that of anti-Vβ5, and the same effects were observed in mature lymph node T cells from OT-I TCR transgenic mice (40) bred onto the TAP-1 knockout background (41). We cultured the thymocytes with anti-Vα2, anti-Cβ, or anti-Vβ5, with or without the inhibitors herbimycin A (inhibitor of the Src family kinases) (41) or PP1 (a somewhat more specific Src family inhibitor, particularly of Lck and Fyn) (38). Anti-Vβ5 effectively modulated expression of all TCR components measured (Cβ, Vβ5, and Vα2) (Fig. 3A and data not shown), whereas anti-Vα2 did not cause down-modulation of Vα2 or TCR in general (Fig. 3B and data not shown). Anti-Cβ gave similar down-modulation to that of anti-Vβ5, and the same effects were observed in mature lymph node T cells from OT-I TAPα/−/− mice (data not shown). Addition of herbimycin A and PP1 to the anti-Vβ5-treated thymocytes rescued TCR from down-modulation (Fig. 3, C and D). The drugs had no effect on cell surface TCR expression of cells incubated with anti-Vα2, as expected (data not shown), demonstrating that neither drug was toxic for the cells under these conditions. These data confirm previous results that the α-chain is regulated differently from the β-chain upon ligation, and that stimulation through Vα results in retention of Vα on the cell surface, whereas stimulation through Vβ results in TCR down-modulation (11). They also confirm that protein tyrosine kinases of the Src family are required to mediate signals leading to TCR down-regulation.

**Both anti-Vα and anti-Vβ cross-link TCR**

However, the results of Fig. 3 are relevant only if both anti-Vα and anti-Vβ Abs are equally effective at cross-linking TCR. Therefore, we performed a capping experiment (Fig. 4). We isolated thymocytes from an OT-I/TAPα/−/− mouse and incubated them with either biotinylated anti-Vα2 or anti-Vβ5 Abs. FITC-labeled streptavidin was added as the cross-linking agent, and the thymocytes were incubated at 37°C. Capping of the fluorescent label was visualized by microscopy. Both anti-Vα2 and anti-Vβ5 were able to induce the formation of a cap, from which we conclude that they are both competent to cross-link TCR.
As in /H9252 V Thymocytes were incubated alone (solid line) or with anti-V after TCR activation (17, 18, 21, 22). Loss of c-Cbl leads to up-regulation of TCR-mediated signaling and is a major target of phosphorylation. Therefore, the inability of anti-V to down-modulate TCR is not explained by their affinities. It was important to demonstrate that the ability of anti-V5, as well as the inability of anti-Vα2, to down-modulate TCR was not due to anti-V5 having a higher affinity than anti-Vα2. Therefore, we measured the binding of anti-TCR Abs to soluble OT-I TCR using Biacore. Biotinylated anti-C5, anti-Vα2, anti-Vα11, and anti-Vβ5 were immobilized on a streptavidin-coated sensor chip, and soluble OT-I TCR was pumped over the surfaces (Table II). As expected, there was no detectable binding to the anti-Vα11 surface. Both anti-Vβ5 and anti-Vα2 bound OT-I TCR with lower affinity than did anti-C5, mainly due to the much slower off-rate for this mAb. The off-rates for anti-Vα2 and anti-Vβ5 were very close, but the affinity of anti-Vβ5 was ~5-fold lower than that of anti-Vα2, primarily due to the faster on-rate of anti-Vα2. Therefore, the inability of anti-Vα2 to down-modulate TCR is not caused by its having a lower affinity than anti-Vβ5.

c-Cbl in α-chain allelic exclusion

The adapter protein c-Cbl is involved in negative regulation of TCR-mediated signaling and is a major target of phosphorylation after TCR activation (17, 18, 21, 22). Loss of c-Cbl leads to up-regulation of TCR expression on immature DP thymocytes and to inhibition of stimulation-induced TCR degradation (21, 22, 28). To investigate its potential role in TCR α-chain allelic exclusion, c-Cbl-deficient mice were tested for Vα expression. As expected (21, 22), the c-Cbl−/− mice showed increased TCR levels in what are normally the TCRlow DP cells (Fig. 5A). Cell surface TCRs from c-Cbl−/− thymocytes were stained with the different available anti-Vα Abs and compared with those from control c-Cbl+/+ littermates. Dual α-chain expression on the cell surface of immature thymocytes from c-Cbl−/− mice was clearly increased in comparison to c-Cbl+/+ mice (Fig. 5B). These data demonstrate a role for c-Cbl in allelic exclusion of α-chain.

To test for a role for lysosomal degradation in allelic exclusion, we compared beige mutant mice (B6 bg/bg ) with B6. The beige mutation causes a defect in lysosomal transport (42). There was no detectable effect on α-chain exclusion (data not shown).

Discussion

The mechanism leading to the down-regulation of one α-chain on the surface of thymocytes undergoing positive selection (phenotypic allelic exclusion) is still unclear. Several models have been proposed to resolve this question (2). In this study, we tested and compared predictions of the different models, clearly refuting one class of model and supporting another.

Differential TCR down-modulation by anti-Vα and anti-Vβ is not explained by their affinities

Anti-Vα2 and anti-Vβ5 Abs are equally effective at cross-linking TCR. A single-cell suspension of OT-I TAP+/− thymocytes was prepared. Thymocytes were incubated with biotinylated anti-Vα2 or anti-Vβ5 Abs followed by staining and cross-linking with streptavidin-FITC. A patchy ring of FITC staining resulting from TCR aggregation appeared on OT-I thymocytes before incubation at 37°C (A). After 2 min at 37°C, cap formation was observed in 35% of the thymocytes treated with either anti-Vα2 (B) or anti-Vβ5 (C) Abs. A representative experiment of three is shown.

FIGURE 3. Src kinase inhibitors block TCR down-modulation induced by anti-Vβ (but not anti-Vα) cross-linking Abs. Thymocytes from OT-I TAP+/− mice were isolated and incubated for 18 h in the presence or absence of immobilized TCR cross-linking Abs and herbimycin A or PP1. A, Thymocytes were incubated alone (solid line) or with anti-Vβ5 (dashed line) for 24 h and then washed and stained for expression of TCR with anti-Cβ-FITC. B, Thymocytes were incubated alone (solid line) or with anti-Vα2 (dashed line), washed, and stained for expression of TCR with anti-Cβ-FITC. C and D, As in A, except that anti-Vβ5-treated cells were incubated with herbimycin A (C) or PP1 (D) (bold lines). A representative experiment of four is shown. Similar data were obtained with staining with anti-Vα2-FITC or anti-Vβ5-FITC in place of anti-Cβ (data not shown).

FIGURE 4. Anti-Vα2 and anti-Vβ5 Abs are equally effective at cross-linking TCR. A single-cell suspension of OT-I TAP+/− thymocytes was prepared. Thymocytes were incubated with biotinylated anti-Vα2 or anti-Vβ5 Abs followed by staining and cross-linking with streptavidin-FITC. A patchy ring of FITC staining resulting from TCR aggregation appeared on OT-I thymocytes before incubation at 37°C (A). After 2 min at 37°C, cap formation was observed in 35% of the thymocytes treated with either anti-Vα2 (B) or anti-Vβ5 (C) Abs. A representative experiment of three is shown.
Table II. Binding kinetics of OT-I TCR to Abs

<table>
<thead>
<tr>
<th>Ab</th>
<th>( k_{\text{on}} ) (M(^{-1})s(^{-1}))</th>
<th>( k_{\text{off}} ) (s(^{-1}))</th>
<th>( K_d ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Va2 (B20.1)</td>
<td>( 4.91 \times 10^4 )</td>
<td>( 2.03 \times 10^{-3} )</td>
<td>( 4.14 \times 10^{-9} )</td>
</tr>
<tr>
<td>Anti-V(\beta) (MR9-4)</td>
<td>( 1.17 \times 10^4 )</td>
<td>( 2.29 \times 10^{-3} )</td>
<td>( 1.95 \times 10^{-8} )</td>
</tr>
<tr>
<td>Anti-(\beta) (H57-597)</td>
<td>( 2.08 \times 10^4 )</td>
<td>( 2.55 \times 10^{-5} )</td>
<td>( 1.23 \times 10^{-10} )</td>
</tr>
</tbody>
</table>

\( ^\text{a} \) Comparison of OT-I TCR binding to anti-Va2, anti-V\(\beta\), or anti-\(\beta\). Biotinylated forms of these Abs, as well as an irrelevant control (anti-Va11 RR8-1), were immobilized on a sensorchip SA (streptavidin surface). Binding of soluble OT-I TCR was then measured using a Biacore 2000 instrument and analyzed by global curve fitting using BIAevaluation 3.1 software.

Competition between two \(\alpha\)-chain proteins for a limiting supply of \(\beta\)-chain protein was the basis of the dueling \(\alpha\)-chain model (8, 10). In immature thymocytes, the \(\alpha\)-chain is produced at a low level and is very unstable (36). Thus, the \(\beta\)-chain would be in excess, allowing both \(\alpha\)-chain proteins to pair with \(\beta\)-chains and to be expressed on the cell surface (10). However, after positive selection, the \(\alpha\)-chain expression is up-regulated, and the protein is much more stable (37). At that point, the \(\beta\)-chains would become limiting, and only the \(\alpha\)-chain with the higher affinity for pairing to the \(\beta\)-chain would be expressed on the cell surface (10). This type of competition has been suggested previously for mature T cells (7, 43). Therefore, we used two different methods to test this. We increased TCR expression pharmacologically (16), predicting that this would induce competition among \(\alpha\)-chains for \(\beta\) and thus reduce dual \(\alpha\)-chain-expressing cells. We also increased \(\beta\)-chain expression genetically, predicting that it would reduce competition among \(\alpha\)-chains and result in larger numbers of dual \(\alpha\)-chain-expressing cells. The results of both these approaches refuted the predictions of the dueling \(\alpha\)-chain model.

A variation of the dueling \(\alpha\)-chain model proposed that CD3\(\xi\), which is required for stabilization and transport of the TCR:CD3 complex to the cell surface (12), is the limiting factor (2). In contrast to the \(\alpha\)-chain, the \(\xi\)-chain is more abundant in immature thymocytes and is down-regulated in mature thymocytes (36, 37). Thus, when limiting, CD3\(\xi\) could favor stabilization of the \(\alpha\beta\) pair using the \(\alpha\)-chain showing the best affinity for the \(\beta\)-chain. Therefore, we doubled the amount of available CD3\(\xi\) by using a \(\xi\)-chain transgenic mouse on a \(\xi\)-knockout background (39) to see whether we could observe more thymocytes with two cell surface \(\alpha\)-chains. This was not the case, again arguing against the dueling \(\alpha\)-chain model of allelic exclusion.

The selective retention model proposes that the positively selected \(\alpha\)-chain is maintained on the cell surface while the nonselected \(\alpha\)-chain is down-modulated (11). This requires that phenotypic allelic exclusion depends on signaling through the TCR, presumably the same signal as mediates positive selection. Because such signaling requires phosphorylation of CD3 proteins by Lck and Fyn, we cultured adult thymocytes in the presence of the Src family kinase inhibitor PP1, which is selective for these two kinases. This resulted in increased dual V\(\alpha\) expression on the cell surface of TCR\textsuperscript{low} and TCR\textsuperscript{high} cells while the control Tcra hemizygous thymocytes showed no such increase. This indicates that \(\alpha\)-chain allelic exclusion depends on the TCR signaling cascade and is thus an active process, rather than the stochastic competition models tested earlier. It is noteworthy that Lck is down-regulated following positive selection, and it has been suggested that the increase in cell surface TCR expression at this point is a result of reduced Lck signaling (16). Similarly, TCR \(\alpha\)-chain allelic exclusion happens when the TCR level is up-regulated following positive selection (2, 8, 10). We found that Ab-mediated cross-linking of \(\beta\)-chain leads to internalization of the TCR, while ligation of V\(\alpha\) leads to retention of the TCR on the cell surface, thus confirming the result of Boyd et al. (11) of differential signaling through ligation of either of the TCR chains. It was important to prove that the anti-V\(\alpha\) reagent used in these experiments is as high affinity and as capable of cross-linking TCR as the anti-V\(\beta\) reagent. This was indeed the case, suggesting that there is an important difference in signaling depending on how TCRs are cross-linked. Blocking of Src kinase activity with herbinycin A or PP1 inhibited the TCR down-regulation caused by cross-linking of \(\beta\)-chain, without affecting anti-V\(\alpha\) cross-linked cells.
Lck is required for the TCR to be ubiquitinated and degraded following TCR engagement (27). Because c-Cbl functions as a ubiquitin ligase, resulting in targeting of tyrosine-phosphorylated receptors for endocytosis and degradation (23, 24), and inactivation of c-Cbl results in up-regulation of TCR expression in immature DP thymocytes that normally have a TCRlow phenotype (21, 22), we hypothesized that c-Cbl could be involved in maintenance of α-chain phenotypic allelic exclusion. Therefore, we analyzed dual Vα expression on the cell surface of Cbcl-deficient thymocytes, finding that it was increased compared with thymocytes from c-Cbl hemizygous mice. This suggested that c-Cbl is involved in allelic exclusion of Vα by targeting the nonselected α-chain for degradation. It is now known that c-Cbl and Cbl-b are involved in internalization and degradation of stimulated TCRs (28). The mechanism by which the TCR receiving the positive selection signal can be protected from c-Cbl-mediated ubiquitination, whereas the nonselected TCR is ubiquitinated and degraded, is under investigation.

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References