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The Regulated Expression of a Diverse Set of Genes during Thymocyte Positive Selection In Vivo


A signal initiated by the newly formed Ag receptor is integrated with microenvironmental cues during T cell development to ensure positive selection of CD4+CD8+ progenitors into functionally mature CD4+ or CD8+ T lymphocytes. During this transition, a survival program is initiated, TCR gene recombination ceases, cells migrate into a new thymic microenvironment, the responsiveness of the Ag receptor is tuned, and the cells commit to a specific T lineage. To determine potential regulators of these processes, we used mRNA microarray analysis to compare gene expression changes in CD4+CD8+ thymocytes from TCR transgenic mice that have received a TCR selection signal with those that had not received a signal. We found 129 genes with expression that changed significantly during positive selection, the majority of which were not previously appreciated. A large number of these changes were confirmed by real-time PCR or flow cytometry. We have combined our findings with gene changes reported in the literature to provide a comprehensive report of the genes regulated during positive selection, and we attempted to assign these genes to positive selection process categories. The Journal of Immunology, 2004, 173: 5434–5444.
the transcriptional programs of DP thymocytes before undergoing TCR-dependent positive selection (preselection DP thymocytes (Pre-DP)) to those of DP thymocytes that have gone through positive selection (postselection DP thymocytes (Post-DP)). Applying stringent selection criteria, we identified 129 genes differentially regulated during positive selection, including many not previously known to be involved during this stage of thymocyte development. Through the use of gene ontology databases, we have placed these genes into categories according to their known or predicted functional processes. The expression of many of these genes was verified by real-time PCR or flow cytometry. In addition, we report a kinetic expression analysis of some genes regulated during thymocyte development. We have attempted to combine our data with other gene changes reported in the literature to provide a comprehensive list of genes regulated during DP thymocyte positive selection. We discuss some of these gene changes with regard to the major processes of positive selection, including thymocyte survival, cessation of TCR gene recombination (allelic exclusion), migration into a new thymic microenvironment, TCR tuning of the repertoire, which will allow researchers to better explore the roles of specific signaling pathways in thymic selection.

Materials and Methods

Mice

OT-I is a C57BL/6/TCR transgenic strain that expresses a transgenic T-cell receptor specific for the OVA 257–264 peptide in the context of H-2Kb (a class I-restricted MHC molecule) (2). OT-I/TKA mice were generated by breeding OT-I mice to TAP-deficient mice on a C57BL/6 background. All mice were bred and maintained in our colony at the University of Minnesota mouse facility (Minneapolis, MN). All mice were treated in accordance with federal guidelines approved by the University of Minnesota Institutional Animal Care and Use Committee. Mice between 6 and 12 wk of age were used for experiments.

Thymocyte purification

Pre-DPs were isolated by incubation of OT-I/TKA−/−/− TCR transgenic thymocytes (one to two thymi pooled) with anti-mouse-CD8ε-FITC (clone 53-6.7) Ab (BD Pharmingen, San Diego, CA) and anti-FITC magnetic microbeads (10 μl/107 cells) (Miltenyi Biotec, Auburn, CA). The positively labeled fraction was purified by magnetic separation over MACS LS columns (Miltenyi Biotec) according to the manufacturer’s recommendations. Post-DPs were isolated by incubation of OT-I/TKA TCR transgenic thymocytes (three to six thymi pooled) with anti-Kb-FITC (clone Y3) Ab (American Type Culture Collection, Manassas, VA) (22), anti-CD8ε-biotin (clone 2.43) Ab, and then anti-FITC MultiSort magnetic microbeads (20 μl/107 cells) (Miltenyi Biotec). The unlabeled fraction was purified by magnetic separation over MACS LS columns. The anti-FITC beads were removed by enzymatic digestion according to the manufacturer’s instructions and then were incubated with streptavidin magnetic microbeads (10 μl/107 cells), and the positively labeled fraction was purified by magnetic separation over MACS LS columns. Staining, washing, and purifying of cells was performed in MACS buffer (1× PBS, 2 mM EDTA, and 0.5% FCS). Populations were determined to be >95% CD4−CD8−Kb−low pure by flow cytometry (Fig. 1A).

Oligonucleotide microarrays

Target RNAs (biont-labeled cRNA fragments) were prepared following technical recommendations found in the Affymetrix Gene Chip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, purified DP thymocytes were lysed using syringe-and-needle homogenization followed by further homogenization over QiaShredder columns (Qiagen, Valencia, CA). Total RNA was isolated using the RNeasy Mini Kit (Qiagen), including on-column DNase digestion (RNase-free DNase Set; Qiagen) following the manufacturer’s recommendations. Double-stranded cDNA was synthesized from at least 6 μg of total RNA using a T7- (dT)24 oligomer (5′-GCCCACTGTTAGTATACGACTATAGGAGGCGG- (dT)24-3′; GENSET, San Diego, CA) with the SuperScript Choice System for cDNA Synthesis (Invitrogen Life Technologies, Carlsbad, CA). Double-stranded cDNA was extracted with phenol/chloroform using Light Phase Lock Gels (Eppendorf Scientific, Westbury, NY) and was ethanol precipitated. Biont-labeled cRNA targets were produced by in vitro transcription of double-stranded cDNA using the BioArray High Yield RNA Transcription Labeling kit (T7; Enzo Diagnostics, Farmingdale, NY) and then cleaned up over RNeasy columns (Qiagen). Finally, 20 μg of biotinylated cRNA was fragmented in 1× RNA Fragmentation Buffer (5× is 200 nM Tris-acetate [pH 8.1], 500 mM KOAc, and 150 mM MgOAc at 94°C for 35 min, followed by cooling on ice. Fifteen micrometers of the fragmented biotinylated cRNA targets were hybridized to the MG_U74 murine gene chips (Affymetrix) for 16 h at 45°C in the GeneChip Hybridization Oven 640 (Affymetrix). Washing was performed using the automated GeneChip Fluidics Station 400 (Affymetrix), and the arrays were immediately scanned on an Agilent GeneArray Scanner (Affymetrix). All hybridizations, washings, and data collections were performed at the Biomedical Image Processing Lab/Lab Advanced Genetic Analysis Center (University of Minnesota). Four independent preparations of OT-I/TA−/−/− Pre-DP and OT-I Post-DP probes were hybridized to four MG_U74A gene chips (two of these were version 1 chips and two were version 2 chips). Only two preparations were hybridized to the MG_U74B2v and MG_U74C2v gene chips.

Identification of differentially expressed genes

Microarray expression results were analyzed using Microarray Suite 5.0 software according to manufacturer’s recommendations (Affymetrix). For consistency, all MG_U74Av1 and v2 chips were analyzed as v1 chips. The MG_U74Av1 mask (Affymetrix) was applied to all A chips to mask out internal probe errors generated by the manufacturer on the v1 A chips. A total of 2,611 probe sets were masked out; thus, a remaining 9,977 probe sets on the A chips were analyzed, along with 12,411 and 11,868 probe sets on the B and C chips, respectively. Therefore, a total of 34,256 probe sets were analyzed. Each chip was scaled to an overall target intensity value of 1,500 to correct for minor differences in overall chip hybridization intensity and to allow comparison between chips. Resulting average scaling factors were as follows: Pre-DP (A), 56; Post-DP (A), 38; Post-DP (B), 196; Post-DP (B), 75; Pre-DP (C), 316; and Post-DP (C), 167. The 3′/5′ signal ratios for the housekeeping genes β-actin and GAPDH were analyzed as a measure of RNA quality. The average 3′/5′ signal ratios for β-actin and GAPDH, respectively, were as follows: Pre-DP (A), 1.65 and 1.80; Post-DP (A), 1.13 and 1.14; Pre-DP (B), 1.78 and 2.41; Post-DP (B), 1.08 and 1.04; Pre-DP (C), 1.89 and 1.91; and Post-DP (C), 1.18 and 0.95. Following the manufacturer’s guidelines, any 3′/5′ ratio of <3 is an indicator of good RNA quality (Affymetrix). Next, pair-wise comparisons of the experimental chips (OT-I Post-DP) to baseline chips (OT-I/TA−/− Pre-DP) were performed. The resulting chip image data were analyzed as follows. Three parameters were queried for each gene probe set (matched/mismatched) on the chip: presence call (present, marginally present, or absent), signal intensity, and change call (increase, marginal increase, no change, marginal decrease, or decrease). Totals of 7,790 and 9,330 probe sets were expressed and considered present on the Pre-DP and Post-DP arrays, respectively (A, B, and C chips combined). Note, however, that many of the probe sets found on the B and C chips represent redundant genes.

We performed the following selection steps to arrive at a gene list. First, genes with “absent” or “no call” in all samples were removed from the analysis. Second, we included only genes that met statistical significance in differential expression between Pre-DP and Post-DP by Student’s t test (p < 0.05 by two-sided pair-wise Student’s t test, assuming unequal variance). Third, we selected only genes for which fold change in expression was greater than two. Fourth, we selected only genes that were present (average detection, p < 0.05) in the secondary population if the gene was considered increased and only genes with a minimal decrease in intensity that were present in the primary population if the gene was considered decreased. Finally, we selected only genes for which average change calls had a value of p < 0.003 using the Affymetrix algorithm. Additional genes were hand removed from the resulting top list due to very low expression values. We depicted increased fold change as infinite (∞) if the corresponding gene was only present in the Post-DP population, whereas decreased fold changes were depicted as infinite (∞) if the corresponding gene was present in the Pre-DP population. The list of genes was annotated by submitting GenBank accession numbers for each probe set on the arrays to The Dragon Database (Database Referencing of Array Genes Online) website (http://www.pevsnerlab.kennedykrieger.org/dragon.htm). This annotated list includes Unigene identification numbers, titles, and name abbreviations for each gene. (Please note that we have chosen to refer to a more common name for some genes mentioned in this paper.) In addition, Affymetrix probe set ID numbers were submitted to the Affymetrix analysis website (www.affymetrix.com/analysis/index.affx) to obtain Gene Ontology Consortium designations.

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Flow cytometric analysis of thymocyte populations and Abs

Purified thymocytes were analyzed by three-color flow cytometry using anti-K-IFITC, anti-mouse-CD4-PE, and anti-mouse-CD8-allophycocyanin Abs. To confirm protein expression on DP thymocytes, total thymocytes were analyzed by four-color flow cytometry using Abs to the following: clone H-5, protein kinase C (clone H-9258), CD53 (clone OX-79), CD69 (clone H-1.2F3), IL-4Ra (clone murine IL-4R-M1), IL-7Ra (clone SB/14), Ly-6e/Tsal/Scall (clone MTS5), TCR-Vz2 (clone B2.01), PD-1 (clone J43), and CD2 (clone RM2-5) (BD Pharmingen, San Diego, CA); and Bcl6 (clone D-8), Bcl-2 (clone H-5), perforin (clone CL6), and murine early growth response (Egr1; clone C-19) (Santa Cruz Biotechnology, Santa Cruz, CA); and activin receptor-like kinase-1 (ALK-1) (R&D Systems, Minneapolis, MN). We also used CCR9 Ab (gift of S. Uehara and P. Love, National Institutes of Health, Bethesda, MD), human Fc-fusion to CCL19/ECL (to detect CCR7; gift of J. Cyster, University of California, San Francisco, CA), and the lectin peanut agglutinin (Vector Laboratories, Burlingame, CA). Appropriate isotype control Abs were used. Surface staining was performed for 30 min on ice in FACS buffer (1 % PBS, 1 % FCS, and 0.002 % sodium azide). Intracellular staining for protein kinase Cθ required fixation in 1 % paraformaldehyde for 15 min at room temperature, permeabilization in 0.5 % Triton X-100 in PBS for 30 min at room temperature, permeabilization in 100 % methanol for 15 min on ice, and staining in FACS buffer for 30 min. Intracellular staining for Egr1 required fixation in 3 % formaldehyde for 10 min at room temperature, permeabilization in 0.5 % Triton X-100 in PBS for 15 min on ice, and staining in FACS buffer for 15 min at room temperature. Cell events were collected using a FACSCalibur (BD Biosciences, San Jose, CA) and data were analyzed using Flowjo software (Tree Star, Ashland, OR). Additionally, the expression of many of these genes was confirmed in OT-I/TAP−/− fetal thymic organ culture (data not shown) as previously described (23).

Quantitative real-time RT-PCR (qrt-PCR) analysis

Messenger RNA expression of candidate genes was confirmed by qrt-PCR. Complementary DNA was made from the DNase-treated purified RNA of DP thymocytes (described above) using oligo(dt) priming (Invitrogen Superscript II kit; Invitrogen, San Diego, CA). Each qrt-PCR was performed at least in duplicate using the Quantitect SYBR Green PCR Kit (Qiagen). For each primer pair used, a standard curve consisting of seven 2-fold dilutions was performed on whole thymus cDNA or sorted DP cDNA to determine the efficiency of the primer pair tested. The log of the cDNA input vs the second derivative of the cycle threshold (Ct) was plotted to determine the slope. Efficiency (E) was calculated using the following equation:

\[ E = 10^{(-1/Slope)} \]

where 1/Slope = 100% efficiency. All qrt-PCRs were run for 30 cycles on the Smart Cycler thermocycler (Cepheid, Sunnyvale, CA). Automatic melting curve analysis was performed after the final cycle to ensure that only single PCR products were analyzed further. Hypoxanthine phosphoribosyltransferase (HPRT) expression was analyzed as a control in all experiments. The fold change in expression of the genes of interest was determined by comparing their Ct values with those obtained for the HPRT control and using the efficiency that was calculated from the standard curve for each experimental gene. The following equation (described by Pfaffl (24)) was used: Fold Change = \( \left( \frac{1}{E_{\text{exp}} - E_{\text{ctrl}}} \right)^{C_{\text{ctrl}} - C_{\text{exp}}} \), where \( E_{\text{exp}} \) and \( E_{\text{ctrl}} \) were determined by using Primersoft 3 software (http://frodo.wi.mit.edu/cgi-bin/primers31) for each primer set.

Results and Discussion

The gene expression profile of thymocyte positive selection in vivo

To identify genes involved in thymocyte positive selection, we used miRNA microarray analysis to compare the gene expression profiles before and immediately after positive selection. Purified DP thymocytes from OT-I/TAP−/− mice were used as a reference sample because they represent preselection precursors that have not received positive selection signals through the TCR because they lack the TAP transporter molecule that is required for loading peptides onto the surface of MHC molecules (Fig. 1A, left). In OT-I transgenic mice, the majority of DP thymocytes have received positive selection signaling, as evidenced by CD69 up-regulation (data not shown). We show that class I MHC molecules, however, are not up-regulated on the surface of OT-I thymocytes until the latest stage of positive selection consistent with what is observed in normal mice (Fig. 1B). Thus, we sorted CD8+ cells,
but used Kb expression to exclude mature single positive (SP) thymocytes in our purification protocol. The OT-I thymus contains a large population of CD4^+CD8^low cells that are "intermediate" between immature DP and mature SP (25). These cells have not up-regulated Kb yet (Fig. 1B) and, thus, were present in the sorted population that we used for gene array analysis (Fig. 1A, right). Both populations were purified to >95%, and total RNA was used to generate biotinylated cRNA. Complementary RNA from four OT-I/TAP^+/+ populations (Pre-DP) and four OT-I/TAP^+ populations (Post-DP) was hybridized to Affymetrix murine gene arrays, containing 34,256 probe sets. Hierarchical clustering of all data showed clear segregation according to differentiation state (Pre-DP or Post-DP populations; data not shown). From this data set, we focused on 129 genes that showed differential expression between Pre-DP and Post-DP (Figs. 2 and 3). These genes were selected by applying a series of stringent selection criteria (see Materials and Methods), including statistical significance of the presence call, greater than 2-fold change in expression level, and statistical significance of the change call.

Comparison to previous microarray studies

Last year Hoffmann et al. (20) reported gene expression profiles of polyclonal thymocytes at various stages of maturation. Of 127 genes they found to be significantly changed between DP and CD8 SP, 16 were also found on our top gene list, including Tdt, CCR7, Itm2a, Sema4A, CD53, and Ian1. Of course, many genes were not on our top gene list because they compared polyclonal DP to mature SP, whereas we compared TCR transgenic preselection with postselection DP. A more relevant comparison can be made between our study and that of Huang et al. (21). Their experimental design was similar to ours in that they compared the transcriptional program between Post-DP thymocyte populations from two MHC class I-restricted TCR transgenics (F5 and P14) and Pre-DP thymocytes from nonselecting MHC^+^ transgenic mice. However, unlike the Affymetrix microarray approach used here, Huang et al. used dual-labeled spotted cDNA arrays containing a more limited number of ~2700 genes, handpicked for immunological relevance. To directly compare our data with theirs, we converted their log_2-fold change in expression results determined by qrt-PCR or flow cytometry to fold change values. Their data showed that 30 of 2700 genes had greater than a 2-fold change in expression (22). In our experiment, qrt-PCR, flow cytometry, or previously published results validated the microarray fold change expression results of 35 genes of 44 tested (Fig. 3A, B, and C).

Comparison to previous microarray studies

FIGURE 1. Purification of cells for gene array analysis. A, OT-I/TAP^+/+ and OT-I thymocytes were stained for the expression of CD4 and CD8 before and after DP thymocyte purification, as described in Materials and Methods. The percentage of cells in each gate is indicated. B, OT-I thymocytes were stained for the expression of CD4, CD8, and Kb. Histogram overlays show expression of the MHC molecule Kb in OT-I thymocyte subpopulations. Kb is differentially expressed in thymocyte populations from OT-I TCR transgenic mice, with high expression in the most immature CD4^+CD8^- DN thymocyte population, low expression in CD4^+CD8^- DP and CD4^+CD8^low INT populations, and high expression in CD4^+ or CD8^+ SP populations. We used the low expression of Kb in sorting Post-DPs from OT-I mice.

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FIGURE 2. Genes that are differentially expressed during thymocyte positive selection. Genes are categorized into functional categories: growth (A); transcription and translation (B); metabolism (C); intracellular signal transduction (D); receptor signal transduction (E); development and morphogenesis (F); DNA recombination, replication, and repair (G); survival, death, and apoptosis (H); cell adhesion and motility (I); and unknown (J). Directional fold change in expression (decrease or increase) of OT-I Post-DPs compared with OT-UTAP+/− Pre-DPs is indicated. Infinite (×) fold changes indicate that the gene was present in only one of the two DP populations being compared. A total of 120 of the 129 genes found to be differentially expressed by mRNA microarray analysis are shown here (filled bars). (Data for the nine genes not included here are found in Fig. 3B.) Twenty-one additional genes were found to be differentially expressed by qrt-PCR analysis of mRNA expression or by flow cytometric analysis of protein expression (hatched bars).
changed expression between Pre-DP and Post-DP. For IL-2, Rsk1, and Hdac5, qrt-PCR suggested that gene expression was not changed (Fig. 4, hatched bars). For Dap12, qrt-PCR showed a decrease in gene expression, supporting the Affymetrix microarray result. However, for Junb, qrt-PCR confirmed the data of Huang et al., finding a 4-fold increase (Fig. 4, hatched bar). Clearly both methods are not without error in reporting. This emphasizes the need to confirm microarray data by alternative methods. Because these problems are relatively rare, however, microarray analysis can, and does, provide informative and reliable results, as evidenced by confirmation of differential expression by alternative methods.

The kinetics of gene expression changes

Many investigators have used microarray analysis to provide information about the kinetics of gene expression changes, particularly after the onset of receptor ligation. We chose not to do this here. Instead, we show the kinetic analysis of a subset of genes whose changes could be monitored by flow cytometry (Fig. 5).

FIGURE 3. Validation of microarray data. Gene expression fold change between OT-I/TAP−/− Pre-DPs and OT-I Post-DPs determined by microarray analysis, qrt-PCR (mRNA expression), or flow cytometry (protein expression) is indicated. The SD of the average fold change was used to evaluate qrt-PCR and flow cytometry data. Literature references are indicated if the gene was previously described to be regulated during thymocyte positive selection. Listed are 44 genes found by microarray analysis in this report (A and B); qrt-PCR or flow cytometry results for nine of these did not support microarray results (B). Also listed are 11 genes not on our top gene list, but that were validated by another method (C), and 14 genes found by the microarray analysis by Huang et al. (D) (21). Fold changes marked with an asterisk were not statistically significant according to our selection criteria (thus, were not on our top gene list). Infinite (∞) fold changes indicate that the gene was statistically called “present” in only one of the two populations being compared.
determined by flow cytometry on the following gated thymocyte subsets: OT-I/TAP<sup>−/−</sup> Pre-DP, OT-I Post-DP, OT-I INT, and OT-I CD8<sup>SP</sup>. The average percentages of maximum values (mean fluorescence intensity minus isotype control mean fluorescence intensity) from at least three independent experiments are plotted with error bars indicating the SD of the mean. The genes are separated into four distinct kinetic patterns of expression: increase (A), delayed increase (B), transient increase (C), and decrease (D).

**FIGURE 5.** Kinetic analysis of selected genes regulated during thymocyte development. OT-I/TAP<sup>−/−</sup> and OT-I thymocytes were stained for the expression of CD4 and CD8. Protein expression of selected genes was showed a general decrease during selection, as did the IL-4Rα served (Fig. 5). CCR9 and the lectin peanut agglutinin binding in vivo data we show in Fig. 5. Four general patterns were observed: (A) mean. The genes are separated into four distinct kinetic patterns of expression: increase (A), delayed increase (B), transient increase (C), and decrease (D).

To further analyze the biological relevance of our findings, we classified the genes based on function: survival/death/apoptosis, cell adhesion/motility, development/morphogenesis, growth, metabolism, DNA recombination/replication/repair, intracellular signal transduction, receptor signal transduction, transcription/translation, and unknown (Fig. 2). We assigned these categories using Gene Ontology information (www.geneontology.org). Notice that a number of these physiological processes are critical to the outcomes of thymocyte positive selection: survival, cell adhesion/motility, DNA recombination, signal transduction resulting in TCR tuning, and changes in cell growth and development resulting in terminal differentiation to a single T cell lineage. The remainder of the paper will discuss gene expression results in the context of the five outcomes of thymocyte positive selection outlined in the introduction.

Survival signals received by developing thymocytes during positive selection prevent programmed cell death and allow the thymocytes to continue along their maturation pathway. The antiapoptotic factor Bcl-2 is thought to play a role in DP thymocyte survival, because it has been found to be up-regulated during this stage of T cell development (38). Although our microarray analysis reported only a 1.6-fold change in Bcl-2 mRNA expression during thymocyte selection, flow cytometry analysis revealed a 3-fold up-regulation of Bcl-2 protein expression in Post-DP compared with Pre-DP thymocytes (Figs. 2H and 3C), which is consistent with previously published results (39–41). Additionally, kinetic analysis of Bcl-2 protein expression in developing thymocytes revealed a gradual increase in expression throughout positive selection and lineage commitment to CD8<sup>+</sup> SP thymocytes (Fig. 5A). This suggests that as a thymocyte matures, the need for Bcl-2 becomes increasingly important in maintaining survival signals. The antiapoptotic factor Bcl-xL, a Bcl-2 homologue, is also expressed in DP cells and may provide survival signals for immature DP cells to survive until positively selected (31, 33). Microarray analysis revealed a significant decrease in Bcl-xL mRNA expression after positive selection consistent with previous reports (Figs. 2H and 3A) (32).

Of particular interest is the gene programmed cell death 1 (PD-1), which was not expressed in Pre-DP thymocytes but was expressed in Post-DP thymocytes according to microarray analysis (Fig. 2H). This expression pattern during positive selection was confirmed by flow cytometric analysis of protein levels (Figs. 3A and 5A). Nishimura et al. (43) reported that only a small percentage of B6 fetal thymic organ cultured DP thymocytes express low levels of PD-1. Because the majority of DP thymocytes in B6 mice are in a preselection stage (Pre-DP), these findings can be reconciled with our data showing no PD-1 expression in Pre-DP thymocytes, but high PD-1 expression in Post-DP thymocytes (Figs. 2H and 3A). PD-1-deficient mice crossed to an HY or 2C TCR transgenic strain exhibit a reduction in CD8<sup>+</sup> SP thymocytes generated despite an increased DP population, suggesting a role for PD-1 in controlling the threshold of positive selection (44). Although an additional report using 2C/Rag2<sup>−/−</sup>/PD-1<sup>−/−</sup> mice found a thymic phenotype indicative of increased negative selection, both studies clearly indicate a role for PD-1 in thymocyte selection (45).

The regulated expression of several other molecules may be important for thymocyte survival after positive selection. We found an 8-fold up-regulation of the GTP binding protein Ian1 in Post-DP thymocytes (Figs. 2H and 3A), confirming previous differential display results that also found Ian1 up-regulated in positively selected DP thymocytes (46). The role of Ian1 in thymocyte development is unclear; however, the down-regulation of the pathogen-induced plant protein Aig1, a homologue of Ian1, resulted in programmed cell death, raising the possibility that the regulated expression of Ian1 during thymocyte development is important for survival (46).

After positive selection signals, the down-regulation of apoptosis-related genes, such as Cerk and Tcl-30, may be important for continuing thymocyte survival and for conferring resistance to steroid-mediated cell death. A role for the ceramide kinase gene Cerk in cell apoptosis has been implicated in programmed cell death in Arabidopsis (47), suggesting that the 3-fold decrease in expression...
in Post-DP thymocytes may be important for continued thymocyte survival (Figs. 2A and 3A). Tcl-30 expression in DP thymocytes correlates with the subpopulation of DP thymocytes destined to undergo glucocorticoid-mediated apoptosis (48); thus, the observed 3-fold decrease in Tcl-30 expression in DP thymocytes may reflect differentiation out of the glucocorticoid-sensitive state (Fig. 2H).

**Regulation of migration and adhesion during thymocyte positive selection**

The ability of thymocytes to migrate within particular regions of the thymus is critical for their maturation (49). This migration typically involves the activation of adhesion molecules that interact with the actin cytoskeleton and directional cues that are either attractive or repulsive in nature. We found a large number of changes in gene expression of chemokine receptors, cytoskeletal molecules, and other factors known to regulate cell motility in other systems (Figs. 2I and 3A).

Several chemokine receptors have been reported to increase (CCR4 (50, 51) and CCR7 (52)) or decrease (CCR9 (53) and CXCR4 (50)) with positive selection. Expression patterns of the chemokine ligands for these receptors are consistent with a potential role in thymocyte migration; TARC (ligand for CCR4) and ELC/SLC (ligand for CCR7) are expressed in the medulla, whereas thymus-expressed chemokine (TECK; ligand for CCR9) and SDF-1 (ligand for CXCR4) are expressed in the cortex.

Consistent with these data, we found that CCR7 expression was increased 11-fold upon selection (Figs. 2I and 3A). Interestingly, this molecule was recently shown to be functionally important both for migration to the medulla (52) and for emigration from the thymus (54). We did not observe a significant decrease in CXCR4 gene expression (1.6-fold decrease; data not shown), despite the fact that Suzuki et al. (50) reported more intense CXCR4 expression in the cortex than in the medulla, by in situ hybridization. However, we note that mouse medullary thymocytes remain SDF-1 responsive (51) and that human thymocytes do not lose CXCR4 expression when assessed by flow cytometry, suggesting that perhaps the more intense CXCR4 signal seen by in situ hybridization reflected the greater cellular density of the cortex. CXCR4 was also not included on our top gene list, in contrast with two reports showing increased CXCR4 mRNA after positive selection (50, 51). Although our microarray data showed a 4.5-fold increase, the statistical significance of the presence call was insufficient for inclusion in our data set (data not shown). This may be because of the low and transient expression of CXCR4 mRNA after selection (50).

A number of reports demonstrated expression of CCR9 on DP thymocytes (53, 55, 56). Its ligand, TECK, is highly expressed on cortical epithelial cells as well as medullary dendritic cells. After positive selection, DP thymocytes gradually lose surface expression of CCR9 (53). We found that CCR9 mRNA (data not shown) and protein expression did not change between Pre-DP and Post-DP populations, but were dramatically reduced by the SP stage (Fig. 5D), suggesting that CCR9 interactions may be transiently important in aiding migration of positively selected thymocytes into the medulla. Interestingly, Uehara et al. (53) showed that although DP cells express CCR9 before selection, they are not responsive to the CCR9 ligand, TECK, until after TCR signaling. In this regard, it is interesting that we observed the up-regulation in DP cells of a regulator of G-protein signaling family members, Rgs3 (Figs. 2I and 3A). The regulated expression of Rgs family members, including Rgs3, after Ag signaling through the BCR has suggested a role for Rgs3 in B cell migration within the lymphoid compartment (57), and thus a similar role may exist in thymocytes that have received positive selection signals through the TCR resulting in Rgs3 up-regulation.

Interestingly, a number of cytoskeleton-related molecules were up-regulated (e.g., Add3, cytip, l-plastin, calponin 2, neuropin, utrin, and ND1-S) or down-regulated (e.g., Mtap4 and Fibulin 2) after thymocyte positive selection (Figs. 2I and 3A), suggesting a potential reorganization of cytoskeletal properties in selected thymocytes.

Intriguingly, we found changes in a number of genes that are associated with axonal migration, specifically semaphorin 4A, plexin D1, and Chl1 (Figs. 2I and 3A). The interaction of semaphorins with their plexin receptors mediates repulsion and attraction cues for axonal migration by linking semaphorin signals to Rho-like GTPases in the cytoskeleton (58). Sema4A is just one member of the semaphorin family that has recently been identified as a potential regulator of immune responses (59). The neural cell adhesion molecule “close homologue of L1,” or Chl1, promotes integrin-dependent cell migration of neurons toward extracellular matrix proteins, and thus may have a similar role in aiding thymocyte migration in the thymus (60).

**Regulation of TCR recombination during thymocyte positive selection**

Positive selection is a checkpoint for proper αβTCR structural assembly. Moving past “GO” in positive selection reflects a productive interaction between the αβTCR and MHC, thus providing cellular communication that a functional receptor has formed. The cessation of further recombination is essential at this stage because the structure of the TCRα locus is such that a primary rearrangement can be replaced by a secondary one, if productive. Thus, the cessation of TCR recombination preserves the TCR specificity of each thymocyte, ultimately protecting against autoimmune. The further recombination of TCR genes is stopped by down-regulation of genes such as Rag1, Rag2, and Tdt (26, 27, 29, 30) and by the loss of TCRα locus accessibility. Our microarray analysis showed a striking down-regulation of Rag1, Rag2, and Tdt in Post-DP compared with Pre-DP (Figs. 2G and 3A). Regarding locus accessibility, a number of transcription factors were induced or repressed during this transition, including ones that could be involved in chromatin remodeling (see Transcription factors regulated during thymocyte positive selection). Such genes have the potential to regulate TCRα locus accessibility. In addition, we observed increased expression of Egr1 (1.8-fold increase; Figs. 2B and 3D), which has been reported to result in increased Id3 expression in DP thymocytes (61). Id proteins negatively regulate the E2 family of basic helix-loop-helix transcription factors, which were shown to result in increased Ag receptor gene accessibility in cell lines (61) and thus could be playing a role in locus accessibility. Our data showed a statistically significant increase in Id3 gene expression between Pre-DP and Post-DP, although it was only 1.7-fold (data not shown).

**Regulation of TCR tuning during thymocyte positive selection**

The most widely accepted model for thymocyte selection proposes that low-affinity self-peptide-MHC/TCR interactions will lead to positive selection, whereas high-affinity interactions will lead to negative selection. Then, as T cells move to the periphery, their TCR sensitivity is tuned down so that mature T cells are no longer sensitive to low-affinity self-peptide interactions, yet they have the potential to become activated by high-affinity foreign peptide interactions. Davey et al. (62) provide evidence that preselection thymocytes are more sensitive to TCR ligation than are mature T cells. A number of molecules from our data set may potentially be...
involved in such tuning, including CD5 and the B and T lymphocyte-associated gene (BTLA) (Figs. 2E and 3C). It is thought that CD5 acts as an inhibitor of TCR signaling and may be important in ensuring appropriate TCR tuning after positive selection because CD5-deficient mice show a preferential shift from positive to negative selection (63). In addition to CD5, we found a 6-fold up-regulation in BTLA expression in Post-DPs (Figs. 2E and 3C). Although BTLA-deficient mice do not exhibit a block in T cell development, T cells are hyperresponsive to anti-CD3 Ab stimulation, suggesting that BTLA may act as a negative regulator of T cell activation after thymocyte positive selection signals (64).

**Regulation of lineage commitment during thymocyte positive selection**

The act of down-regulating the expression of one of the TCR coreceptors is important in the transition from CD4+CD8+ DP thymocytes to CD4+ or CD8+ SP thymocytes during the process of lineage commitment. During this step, thymocytes undergo major genetic changes to allow different effector functions of mature CD4+ helper or CD8+ cytolytic T cells. It is predicted that gene changes that could influence chromosomal remodeling of cytokine genes or effector molecules occur as thymocytes mature into the CD4+ or CD8+ T cell lineages and could be present among the genes we present here. Our approach did not allow us to discriminate which genes would be differentially expressed in selected MHC class II-restricted CD4+ thymocytes compared with class I-restricted CD8+ SP cells. However, one might expect, based on the coreceptor reversal model of lineage commitment, that lineage-specific gene changes would not occur until later stages in selection (65). Consistent with this, data from Huang et al. (21) suggest that only a small number of lineage-specific gene changes were observed early in positive selection. In addition, we compared our OT-I Post-SP data set with Post-SPs isolated from OT-II and OT-I/IdLGFl (66) transgenic mice (that develop into CD4 SP). We found fewer than 20 differences between these populations (data not shown). It may be that a more informative view of lineage commitment signals will be found through a comparison of CD4+CD8low INT thymocyte populations in class I- vs class II-restricted TCR transgenics. This would include cells that have undergone positive selection, but that have not yet matured to the CD4 and CD8 SP stage of development.

**Transcription factors regulated during thymocyte positive selection**

A large number of genes on our top gene list are transcription factors (Fig. 2B). Some of these have previously been known to change during positive selection, such as Ets2 (67) and Myb (68) (Figs. 2B and 3A). Several of the genes that were decreased during selection are known or putative transcriptional repressors: Zfp88, Bcl6, Klf3, and Befa2t3h (Figs. 2B and 3A). Both Zfp288 and Bcl6 contain BTB/POZ domains that recruit histone deacetylases. Klf3 (basic Kruppel-like factor) is a transcriptional repressor that binds the C-terminal binding protein corepressor (69). Finally, Befa2t3h (ETO-2) is a runx domain-containing transcription factor that binds histone deacetylases (70). Of the genes increased, we found several genes encoding zinc finger (Mif2, Klf2) or leucine zipper (Ma1b, Ltzfl2, Bzw2) domains (Figs. 2B, 3A, and 3D). An interesting novel gene (Mm.135235) that was up-regulated during selection is a transcription factor with Chromo, SNF2, and helicase domains, suggesting a potential involvement in chromatin remodeling.

**Other gene changes**

Other gene changes of interest include three genes involved in regulation of TGFβ/bone morphogenetic protein signaling: Endoglin (Figs. 2F, 3A, and 5A), ALK-1 (Figs. 2F, 3C, and 5B), and Smad7 (Fig. 2F). Endoglin and ALK-1 may cooperate to convert the TGFβ receptor into a Smad 1/5/8-dependent signaling receptor, as opposed to the classic Smad 2/3 pathway (71–73). Although there is some evidence that TGFβ/bone morphogenetic protein family members play a role at the β-selection checkpoint (74–77), there is no information as to their role during positive selection or lineage commitment.

It is worth noting that we observed changed expression of seven nonclassical class I genes (five T region genes and two Q region genes; data not shown), consistent with previous reports showing that expression of these genes is developmentally regulated in the thymus (78). In addition, the expression of four Ig genes (data not shown) was decreased during selection. We assume this reflects either hybridization irregularity or “cross-lineage” germline transcription of B cell-specific genes in T cells, as has been observed for Ig-β (79). Finally, we noted an increase in the TAP1 gene, but not the TAP2 gene (data not shown), as would be expected because we were comparing TAP1-deficient cells with wild-type cells.

In conclusion, the data presented in this paper show a large number of novel gene changes observed during thymocyte positive selection. Confirmation of a total of 35 genes through qrt-PCR, flow cytometry, or previously published reports supports the overall validity in the gene expression results generated by microarray analysis reported here. It is our hope that the gene changes documented in this paper will serve as an important resource for further characterizing signaling pathways and molecular targets during thymic selection of the T cell repertoire.

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**References**


