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Transcriptional Analysis of Clonal Deletion In Vivo[1]

Troy A. Baldwin and Kristin A. Hogquist[2]

Engagement of the TCR on $CD4^+CD8^+$ thymocytes initiates either a program of survival and differentiation (positive selection) or death (clonal deletion), which is dictated in large part by the affinity of the TCR for self-peptide-MHC complexes. Although much is known about the factors involved in positive selection, little is understood about the molecular mechanism leading to clonal deletion. To gain further insight into this process, we used a highly physiological TCR transgenic mouse model to compare gene expression changes under conditions of nonselection, positive selection, and negative selection. We identified 388 genes that were differentially regulated in negative selection compared with either nonselection or positive selection. These regulated genes fall into many functional categories including cell surface and intracellular signal transduction, survival and apoptosis, transcription and translation, and adhesion and migration. Additionally, we have compared our transcriptional profile to profiles of negative selection in other model systems in an effort to identify those genes with a higher probability of being functionally relevant. These included three up-regulated genes, *bim*, *nur77*, and *ian1*, and one down-regulated gene, *lip1*. Collectively, these data provide a framework for understanding the molecular basis of clonal deletion. ***The Journal of Immunology,*** **2007, 179: 837–844.**

Selection events in the thymus are critical for shaping the peripheral T cell repertoire. Whereas positive selection ensures the peripheral T cell pool is able to respond to a wide variety of foreign Ags in a self-MHC-restricted fashion, negative selection maintains T cell self-tolerance. Clonal deletion is the predominant form of negative selection, although anergy and receptor editing have been described, and occurs when an immature $CD4^+CD8^+$ double-positive (DP)[3] thymocyte expresses a TCR with high affinity for self-peptide MHC (1). This high-affinity interaction results in apoptosis. DP thymocytes with a low-to-moderate affinity TCR are positively selected and initiate a survival and differentiation program to become $CD4^+$ or $CD8^+$ single-positive thymocytes (2). Precisely how the same TCR can initiate either a program of survival and differentiation or death is still a matter of great debate; however, recent experiments have suggested that quantitative differences in TCR-coupled signaling pathways can lead to these qualitatively distinct outcomes (3). Therefore, it is of great interest to determine what transcriptional programs are induced by positive vs negative selection in hopes of identifying molecules critical for these outcomes.

Currently, little is known regarding the molecular events underlying clonal deletion. Although TCR engagement and many membrane proximal signal transduction molecules including the lymphocyte-specific protein tyrosine kinase (Lck), Zap70, and the linker for activation of T cells are likely required for both positive and negative selection, specific pathways leading to negative selection are somewhat unclear (2). Differential MAPK signaling has been demonstrated to be important for the positive vs negative selection choice with a short, high-intensity burst of ERK activation leading to clonal deletion, whereas sustained ERK signaling resulted in positive selection (4–6). The activation of JNK and MAPK14 (p38) pathways also appear to play a role in clonal deletion (7–9). Recently, it was demonstrated that negative selection signals compartmentalize MAPK signaling to the plasma membrane, whereas positive selection results in MAPK signaling throughout the thymocyte, providing yet another clue as to how the TCR can discriminate between positive- and negative-selecting ligands (10). In addition, the germinal center kinase misshapen-like kinase 1 is necessary for negative selection, but does not appear to influence positive selection (11). How misshapen-like kinase 1 links the TCR to clonal deletion is currently unclear; however, it appears to involve the induction of BCL2-like 11 (Bim) (11).

Two molecules most closely linked to clonal deletion are the BH3 only Bcl-2 family member Bim and the nuclear steroid orphan receptor Nur77. $Bim^{-/-}$ thymocytes are refractory to Ag-specific negative selection in vivo, indicating that the presence of Bim is required for negative selection (12); however, it is currently unclear whether or not an increase in Bim expression is necessary. Many studies have demonstrated an increase in Bim mRNA and protein levels following a negative-selection stimulus (13, 14), although one study found no increase in Bim protein levels (15). Bim can also be regulated posttranslationally during negative selection, although it is unknown what effect this regulation has on clonal deletion (15). Transgenic overexpression of a Nur77 dominant-negative protein also impaired in vivo Ag-specific clonal deletion (16). Although $Nur77^{-/-}$ mice showed no defect in clonal deletion, it is suspected that other family members including Nor1 can compensate (17). The precise mechanism by which Nur77 exerts its proapoptotic activity is unclear; however, Nur77 constructs that reduced its transcriptional activity also reduced its ability to induce negative selection (18).

To gain more insight into the molecular mechanism underlying clonal deletion, we analyzed the transcriptome of HY^{cd4} DP thymocytes that received either no signal (nonselection), or positive or negative selection signals in vivo. Data generated in this fashion

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[3] Abbreviations used in this paper: DP, $CD4^+CD8^+$ double positive; qRT-PCR, quantitative real-time PCR; MHC II, MHC class II; Bim, BCL2-like 11; PD-1, programmed cell death 1.

allowed a global analysis of selection and differentiation that occurs during thymic development. The HY^{cd4} system provides a highly physiological model for thymic development and its benefits include the appropriately timed expression of TCRα and endogenous expression of ubiquitous self-Ags (19). Additionally, through the use of mixed bone marrow chimeras, we ensured the maintenance of normal thymic architecture through reduced precursor frequency. We found 870 unique genes were regulated 2-fold or more when comparing one DP thymocyte population to either of the other two. Furthermore, 388 genes were specifically regulated when comparing negative selection to both nonselection and positive selection. Recently, a number of other groups have taken similar approaches to try and identify molecules important for negative selection. We compared our gene list to these recently published lists to identify those common genes that may be key regulators of clonal deletion. Verification by quantitative real-time PCR (qRT-PCR) was performed for those common genes and those whose expression pattern was verified are discussed further in the context of clonal deletion. Collectively, these data provide a basis for understanding the molecular mechanism of negative selection.

Materials and Methods

Mice

C57BL/6 (B6) and C57BL/6 Ly5.1 (B6.SJL) mice were purchased from the National Cancer Institute (Frederick, MD) or The Jackson Laboratory. HY^{cd4} and HY^{cd4} $TCR\alpha^{-/-}$ mice were previously described (19). $HY^{cd4}D^{b-/-}$ mice were generated by breeding HY^{cd4} mice to $D^{b-/-}$ mice purchased from Taconic Farms. All mice were bred and maintained in our colony at the University of Minnesota mouse facility and used between 4 and 12 wk for experiments. All mice were treated in accordance with protocols approved by the University of Minnesota Institutional Animal Care and Use Committee.

Bone marrow chimeras

Bone marrow was harvested from donor animals and T cell depleted using anti-Thy1.2 culture supernatant (clone 30H12) and complement (Cedarlane Laboratories, Hornby, Ontario, Canada). $HY^{cd4}TCR\alpha^{-/-}$ and B6.SJL bone marrow was mixed at a 2:1 ratio and injected into lethally irradiated (1000 Gy) B6.SJL recipients. Six to 8 wk following reconstitution, the thymus was harvested from the animals for cell sorting.

Abs, cell sorting, and flow cytometry

All Abs were purchased from BD Biosciences, eBioscience, or BioLegend. The thymus from $HY^{cd4}TCR\alpha^{-/-}$ male or female chimeric mice or unmanipulated $HY^{cd4}D^{b-/-}$ mice was harvested and thymocyte single-cell suspensions were stained with Abs to CD45.2, CD4, CD8, and either CD69 or T3.70 for 30 min on ice in PBS plus 2% FCS (sorting buffer). Cells were washed twice in sorting buffer before cell sorting. For sorting, the $CD45.2^+$ cells were gated, a $CD4^+CD8^+$ gate was applied, and either $CD69^+$ or $T3.70^+$ cells were collected. Staining for flow cytometry was performed in the same fashion; however, 0.02% sodium azide was added to the sorting buffer.

Oligonucleotide microarrays

Total RNA was harvested from purified thymocytes using the RNeasy midi kit (Qiagen, La Jolla, CA) according to the manufacturer's recommendations (on-column DNase digestion was included). RNA was quantified using Ribogreen (Invitrogen Life Technologies). One hundred to 200 ng of total RNA was used for generating biotinylated cRNA through two rounds of amplication using the Ambion MessageAmp aRNA Amplification kit following the manufacturer's recommendations (Ambion). A total of 20 μg of biotinylated cRNA was fragmented in 1× fragmentation buffer (40 mM Tris-acetate (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate) at 94°C for 35 min. The fragmented, biotinylated cRNA was hybridized to Affymetrix murine 430 2.0 gene chips (Affymetrix), washed, and scanned at the Biomedical Genomic Center (University of Minnesota) following standard procedures. Three independent RNA samples were analyzed.

Microarray analysis

The microarray data were analyzed using GeneSpring GX 7.3.1 software (Agilent). Following the import of Affymetrix CEL files, a new experiment was created and per chip and per gene normalization was performed. Only probes sets with a raw signal value of >50 in at least one of three conditions were kept. Statistical analysis included a one-way ANOVA ($p \leq 0.05$) with a Benjamini and Hochberg false discovery rate multiple-testing correction, and a fold-change (2-fold) cutoff was applied. Additionally, probe sets were excluded that did not have a raw signal value difference of ≥300 in one condition compared with either other condition. If one gene was represented by multiple probe sets, additional probe sets were removed leaving only one representative probe set for each gene.

cDNA synthesis and qRT-PCR

cDNA was synthesized from 25 ng of DNase-treated RNA from FACS-purified DP thymocytes using oligo(dT) and random hexamer priming (SuperScript III kit; Invitrogen Life Technologies). qRT-PCR was conducted with Fast Start SYBR Green Master Mix kit (Roche). Fold changes were calculated using the ΔΔCt method with nonselection HY^{cd4} samples as the baseline sample and *β-actin* as the reference gene. The following primer sequences were used: β-actin, forward, 5′-CTAAGGCCAACCGTGAAAAG-3′, reverse, 5′-ACCAGAGGCATACAGGGACA-3′; Bim, forward, 5′-CGGTCCTCCAGTGGGTATTT-3′, reverse, 5′-AGGACTTGGGGTTTGTGTTG-3′; Gadd45β, forward, 5′-GAGACCTGCACTGCCTCCT-3′, reverse, 5′-CATTGGTTATTGCCTCTGCTC-3′; Ian1, forward, 5′-TCTTGCTCACCAGGAAGGAT-3′, reverse, 5′-AGCGATTCTGGAACTCATGC-3′; $I\kappa B_{NS}$, forward, 5′-GGGCTCTTTCCCATTCTCT-3′, reverse, 5′-CCAGAGAGTCCTCCATCACC-3′; Lip1, forward, 5′-GTATTCACCGAATCCCTCGT-3′, reverse, 5′-TGTCAATGTTTGTGACCCAGTT-3′; and Nur77, forward, 5′-GGCATGGTGAAGGAAGTTGT-3′, reverse, 5′-TGAGGGAAGTGAGAAGATTGGT-3′.

MACS purification and Western blot

Thymocytes from $HY^{cd4}TCR\alpha^{-/-}$ male and female mice were stained with anti-CD69 FITC for 15 min at 6–12°C in MACS buffer (Ca^{2+}- and Mg^{2+}-free PBS, 1% FCS). The cells were washed two times in MACS buffer and anti-FITC microbeads (10 μl per 1×10^7 cells) were added. Following 15 min at 6–12°C, the cells were applied to an equilibrated MACS LS column. The adherent fraction ($CD69^+$) cells were collected and lysed at 500×10^6 cells/ml in lysis buffer (PBS plus 1% Nonidet P-40). A total of 5×10^6 cell equivalents was resolved by SDS-PAGE and Western blotted with anti-Bim (StressGen Biotechnologies), anti-Ian1 (a gift from Dr. H. Jacobs, National Cancer Institute, Amsterdam, The Netherlands) or anti-Erk2 (Santa Cruz Biotechnology) as a loading control.

Results

The gene expression profile of cells undergoing positive or negative selection in vivo

To identify genes involved in the processes of positive selection or clonal deletion, we purified HY^{cd4} DP thymocytes that had received either a positive- or negative-selection signal in vivo. The HY^{cd4} system provides a highly physiological means to model both positive and negative selection in vivo due to the appropriately timed expression of the HY TCR α-chain and endogenous selecting ligand expression (19). To avoid artifacts related to altered thymic architecture in TCR transgenic mice (20) and complexities due to endogenous TCR expression, we isolated HY^{cd4} DP thymocytes from mixed bone chimeras. $HY^{cd4}TCR\alpha^{o}$ male or female bone marrow was mixed with male or female congenic wild-type bone marrow at a 2:1 ratio and injected into lethally irradiated male or female congenic recipients. In the thymus of chimeric mice, TCR transgenic donor cells ($CD45.2^+$) comprised between 40 and 70% of the total thymocytes (data not shown). Six to 8 wk following reconstitution, the animals were harvested and either $CD69^+$ (Fig. 1) or $T3.70^+$ (data not shown) HY^{cd4} DP thymocytes were isolated. RNA from both $CD69^+$ and $T3.70^+$ sorted subsets were subsequently used for generation of labeled cRNA (see below). Purified $T3.70^+CD69^-$ DP thymocytes from $HY^{cd4}D^{b-/-}$ mice were used as a nonselection reference population (the HY TCR is restricted by D^b) (Fig. 1). Three independent

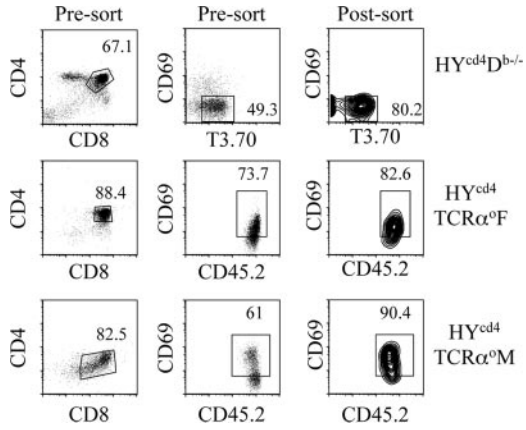

FIGURE 1. Purification of HY^{cd4} thymocytes for transcriptome analysis. Thymocytes from $HY^{cd4}D^{b-/-}$ mice (nonselection), $HY^{cd4}TCR\alpha^{o}F$ chimeric mice (positive selection), and $HY^{cd4}TCR\alpha^{o}M$ chimeric mice (negative selection) were stained with anti-CD45.2, -CD4, -CD8, and -CD69. $CD45.2^{+}CD69^{+}$ DP thymocytes were electronically gated and purified to >80% purity by FACS. Three independent sorts were performed, and representative results are indicated.

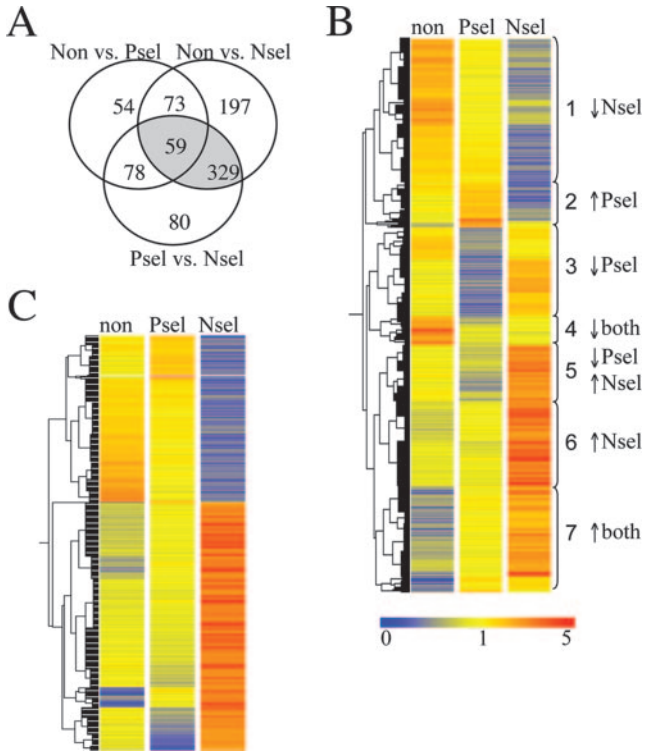

FIGURE 2. Clustering of differentially expressed genes. *A*, A Venn diagram illustrating the overlap of differentially expressed genes for all three possible comparisons. A total of 870 unique genes was identified. *B*, Cluster analysis of the 870 unique genes that were identified as differentially expressed. At least seven unique patterns of expression related to selection are observed for the differentially expressed genes. *C*, Cluster analysis of 388 genes differentially regulated during negative selection (genes highlighted in gray in *A*).

sorts from positively selecting, negatively selecting, and nonselecting animals were obtained. Total RNA was isolated from sorted cells and subjected to two rounds of cRNA amplification and hybridized to Affymetrix mouse 430 2.0 arrays. The data were analyzed using Agilent Genespring software, applying stringent statistical analysis including one-way ANOVA, 2-fold change in normalized expression level, and minimum raw signal value changes. If a gene was identified as differentially expressed by multiple probe sets, duplicate probe sets with similar expression patterns were removed for ease of analysis.

A total of 870 genes was identified as being differentially expressed in one cell population compared with either of the other two (Fig. 2*A*). Cluster analysis of these 870 genes revealed at least seven different expression profiles including genes induced or repressed by negative selection, genes induced or repressed by positive selection, genes induced or repressed by either positive or negative selection, and genes repressed in positive selection while being induced in negative selection (Fig. 2*B*). A list of the 870 differentially expressed genes is presented in supplemental Fig. 1.[4] Many of the genes on this list have previously been demonstrated to be regulated by positive or negative selection, including *Rag2* (21), *pre-TCRa* (22), *CD53* (23, 24), and *Itm2a* (25), to name but a few, confirming the general validity of the approach. Furthermore, we attempted to confirm the differential expression of at least 30 genes on the list of 870 by either flow cytometry or qRT-PCR as a measure of false-positive incidence. Of the 30 genes examined, only 2 were found to display an expression pattern not observed by gene array analysis. The 28 confirmed genes are highlighted in yellow in supplemental Fig. 1 and those that did not confirm are highlighted in gray. Of interest, there was a larger number of genes changed when comparing nonselection to negative selection (658) as opposed to nonselection compared with positive selection (264). This suggests an overall greater similarity between nonselection and positive selection compared with negative selection. In fact, nonselection and positive selection samples clustered in a hierarchical cluster analysis (data not shown). This may reflect the stronger signal inherent to negative selection given the high affinity interaction that drives it. A total of 388 genes (highlighted in gray) was identified as specifically regulated during negative selection compared with both nonselection and positive selection (Fig. 2*A*). Cluster analysis of these revealed two major profiles, genes that were repressed or induced by negative selection (Fig. 2*C*).

The genes specifically regulated by negative selection (388) are presented in supplemental Fig. 2 and were further subdivided into known or suspected functional categories. These categories include cell adhesion/motility, cellular metabolism, development/morphology, DNA recombination/repair/replication, growth, intracellular signal transduction, protein transport, receptor signal transduction, survival/death/apoptosis, transcription/translation, and unknown.

Comparison to other microarray studies

Over the last few years, a number of microarray studies examining gene expression changes induced by negative selection have been reported (26–30). These studies have used many different models of clonal deletion with the physiological relevance of some of the models being questionable. Fig. 3 outlines the different studies examining clonal deletion and provides a description of the model used in that study. We compared our data set to the other published data sets in an attempt to identify common genes. We limited our formal comparison to only those studies that used an in vivo means to deliver the negative-selection signal, either by expression of the negatively selecting ligand or injection of a high-affinity peptide. Although DeRyckere et al. (26) used an in vivo model, they identified only a small number of gene changes, so the comparison with

[4] The online version of this article contains supplemental material.

Study	Baldwin*	Liston*	DeRyckere	Huang	Zucchelli	Schmitz*
model Ag source system	HY^{cd4} (MHCI restricted) ubiquitous self-Ag in vivo	3A9 (MHCII restricted) ins-HEL Tg in vivo	DO11.10 (MHCII restricted) OVA peptide in vivo	$MHC^{-/-}$ DP thymocytes anti-TCRβ/CD2 in vitro	BDC2.5 (MHCII restricted) BCDmi peptide ex vivo FTOC	N15 $Rag2^{-/-}$ (MHCI restricted) VSV8 peptide in vivo
pre-selection	FACS sorted DP from HY^{cd4} $D^{b-/-}$	FACS sorted CD69- TCR- DP	N/A	$MHC^{-/-}$ DP	N/A	FACS sorted DP from N15 $H\text{-}2^{d}$
positive selection	FACS sorted CD69+ or T3.70+ DP (female)	FACS sorted CD4+CD8lo CD69+TCR+ (early 4SP)	FACS sorted DP	MACS sorted DP from various MHCI or MHCII restricted TCR Tg	FACS sorted DP from FTOC	N/A
negative selection	FACS sorted CD69+ or T3.70+ DP (male)	FACS sorted CD4+CD8lo CD69+TCR+ (early 4SP) from ins-HEL	FACS sorted DP 2 or 8h post OVAp injection i.v.	$MHC^{-/-}$ DP stimulated for 2, 4, 8 or 16h with anti-TCRβ/CD2	FACS sorted DP from FTOC 2 or 16h post BDCmi peptide addition	FACS sorted DP 0.5, 1 or 2h post VSV8 peptide injection i.v.

FIGURE 3. Direct comparison of DNA microarray studies examining clonal deletion. Listed are the six studies that examined gene expression changes following negative selection and characteristics (model, Ag source, and type of system) of those studies. Studies that will be formally compared are indicated (∗).

this data set will not be formally presented. Genes of interest identified in the DeRyckere and other studies will be discussed as they arise.

Perhaps the most similar study to ours, in terms of a physiological model, was undertaken by Liston et al. (28). They used a hen egg lysozyme-specific, MHC class II (MHC II)-restricted TCR transgenic mouse engineered to express hen egg lysozyme under the control of the rat insulin promoter (14). With this system, the high-affinity Ag is expressed in an autoimmune regulator-dependent fashion in the thymic medulla. Although the TCR is expressed early in development, the thymocytes presumably do not encounter their high-affinity Ag until they have been positively selected and have trafficked to the medulla. Similar to our study, Liston et al. FACS sorted prepositive and prenegative selection thymocytes and early-positive and early-negative selection thymocytes for comparison. After applying the same 2-fold change cutoff to their data set and removing duplicate probe sets, we directly compared their resultant gene list (188 genes) with ours (388 genes) (Fig. 4*A*). Forty genes were found to be in common between the two studies (21% of the Liston gene list and 10% of our gene list). Interestingly, only 16 of the 40 genes (40%) showed a similar expression pattern in the two data sets. These genes are highlighted in gray (Fig. 4*A*).

We next compared our gene list to that of Schmitz et al. (29). They injected VSV8 peptide into young N15 TCR transgenic mice and FACS sorted DP thymocytes at various times after stimulation. Although this model has the disadvantage of using peptide injection of a TCR transgenic, the thymocytes were likely harvested before nonspecific cytokine and glucocorticoid effects mediated by activation of peripheral Ag-specific T cells (31). We found 67 genes in common between the two studies (29% of the Schmitz gene list and 17% of our gene list) (Fig. 4*B*). All of the common genes displayed an identical expression pattern and are highlighted in gray (Fig. 4*B*).

There are at least two possible, nonmutually exclusive reasons for the apparent lack of similarity between our data set and the Liston data set and the similarity between our data set and the Schmitz data set. The first possibility is the fact that both our study and the Schmitz study used a MHC I-restricted model, whereas the Liston study used a MHC II-restricted model. Another possibility centers on the stage and anatomical location that the DP thymocyte would encounter the high-affinity ligand. In our model and in the Schmitz model, ligand encounter likely occurred in the cortex at the DP stage, whereas in the Liston model the DP thymocytes are required to receive a positive-selection signal and subsequently traffic to the thymic medulla to receive a high-affinity signal. We favor the latter possibility for at least two reasons. First, there have only been a few genes demonstrated to be differentially regulated in DP thymocytes during MHC I vs MHC II selection (most notably *Th-POK* (32, 33)) (27). Second, because in the Liston model, thymocytes first need a positive selection signal they have become a nondividing population—far removed from the cycling double-negative progenitors. In our model and the Schmitz model, the DP thymocytes that encountered high-affinity Ag have just come from the cycling double-negative pool and likely still contain remnants of that transcriptional profile. In fact, a number of the genes not highlighted in Fig. 4*A* are involved in the cell cycle and proliferation.

We next compared the genes that were in common to all three data sets in an effort to identify genes with a higher probability of being functionally important in clonal deletion. Only seven genes were found to be in common with all three data sets. These genes were as follows: ↑ *Bim*, ↑ *G7e*, ↑ *Ian1*, ↓ *Lip1*, ↑ *Mtm1*, ↑ *Nur77*, and ↓ *Rag2*. *Rag2* was excluded from further analysis because it is involved in TCR gene recombination and its repression is known to contribute to allelic exclusion at this stage, and not clonal deletion. qRT-PCR of the remaining six genes was performed in an attempt to verify the microarray data. *Bim*, *Ian1*, *Lip1*, and *Nur77* displayed similar expression patterns to what was observed by microarray analysis (Fig. 5, *A* and *B*). *G7e* showed a 2.6-fold increase in expression from positive to negative selection, but was unchanged between nonselection and negative selection. However, *Mtm1* showed a 1.6-fold decrease from positive to negative selection, in contrast to the 2.7-fold increase on the array (data not shown). The increase in *ian1* expression in HY^{cd4} male over female was not as dramatic as what was observed by microarray analysis; however, the qRT-PCR was conducted with cDNA synthesized from RNA isolated from $HY^{cd4}TCR\alpha^{+}$ as opposed to $HY^{cd4}TCR\alpha^{o}$ DP thymocytes. This may account for the difference between microarray and qRT-PCR data. Additionally, when $HY^{cd4}TCR\alpha^{o}$ female and male DP thymocytes were purified and lysates were probed by Western blot, an increase in both Bim and Ian1 protein levels were also observed (Fig. 5*C*). Therefore, although all three studies identified a large number of gene changes, only a small number of genes were found to be in common.

Examination of the other three data sets not included in the formal analysis above revealed that only *nur77* was consistently identified as being regulated during negative selection. *Bim* was identified by both the Huang and Zucchelli studies as being induced. Given the putative role of both Nur77 (16) and Bim (12) in negative selection, the consistency with which these genes are induced in numerous models of clonal deletion and the lack of numerous other genes being consistently identified (with the exception of programmed cell death 1 (*pd-1*) and *gadd45β*) (see

A

common name	Baldwin neg vs non	Baldwin neg vs pos	Liston neg vs non	Liston neg vs pos
2310010M24Rik	-3.44	-3.38	-2.49	-2.12
Arpp21	-10.50	-4.30	-4.98	2.07
Aurkb	2.07	5.57	-4.11	2.53
Bim (Bcl2l11)	5.67	3.92	6.49	2.68
Brrn1	2.14	3.04	-2.65	2.18
Ccna2	2.36	6.04	-3.61	2.30
Ccnb1	2.36	8.66	-3.79	4.69
Cdc20	2.27	4.95	-2.14	2.79
Cdca5	2.07	9.16	-4.20	2.63
Cenpe	2.28	6.77	-2.21	3.12
Cep55	2.72	9.74	-2.57	5.31
Chek1	2.95	6.03	-2.44	3.53
G7e	3.90	7.77	-2.25	2.33
Fbxl10	3.28	3.17	2.45	2.34
Ian1 (gimap4)	15.76	4.06	2.93	-2.38
Hmmr	2.52	7.46	-2.60	3.91
Il2ra	-5.17	-8.52	2.16	4.75
Kif11	2.36	5.61	-2.48	2.60
Kif2c	2.63	13.19	-2.75	7.52
Lactb	2.88	3.12	4.14	2.46
Lig1	2.07	3.55	-4.18	2.23
Lip1	-4.32	-3.66	-2.89	-2.08
Luzp5	2.86	5.59	-2.60	5.09
Ly6d	-3.23	-2.70	-4.70	2.74
Mad2l1	2.21	3.90	-2.96	2.41
Mtm1	2.69	2.67	2.89	2.02
Nur77 (Nr4a1)	16.06	11.29	10.17	2.09
Plk4	3.22	4.14	-2.41	2.62
Racgap1	2.81	5.10	-3.23	4.29
Rad51	2.12	4.36	-3.44	2.93
Rag2	-7.71	-6.44	-6.02	2.20
Rapgef3	-3.52	-2.43	-4.19	3.42
Rcn	3.07	3.32	2.92	2.97
Rrm1	2.57	3.71	-3.01	2.23
Shcbp1	2.25	8.42	-2.27	4.60
Socs3	-14.08	-5.88	-2.02	-2.88
Swap70	2.29	2.21	2.82	2.69
Trip13	2.90	6.55	-3.58	3.26
Zfp52	6.72	7.77	5.55	2.45
ZWILCH	2.27	4.53	-3.08	2.32

B

common name	Baldwin neg vs non	Baldwin neg vs pos	Schmitz 30' neg vs non	Schmitz 60' neg vs non	Schmitz 120' neg vs non
1110013L07Rik	-2.05	-2.17	-2.87	-4.55	-6.51
1810015C04Rik	5.99	4.32	2.19	3.80	3.91
Acas2l	-3.17	-2.46	-2.00	-4.06	-6.13
Adk	7.94	12.00	1.77	2.39	4.27
Apex1	4.39	2.63	1.38	3.50	3.90
Arl6ip2	-2.90	-2.87	-2.11	-3.76	-4.64
Bcl2	2.98	3.56	6.31	8.75	3.96
Bim (Bcl2l11)	5.67	3.92	13.38	6.65	12.19
Capn3	-4.66	-2.28	-7.33	-10.71	-12.66
Ccnd2	19.21	17.77	1.36	6.35	19.27
Cd8a	-3.02	-2.90	-1.28	-2.03	-3.92
Cd8b	-6.96	-5.45	-1.89	-2.62	-7.51
Cnn2	3.86	2.95	4.65	4.39	2.34
Csda	3.89	7.58	1.25	3.71	7.71
Ctsl	-3.00	-2.72	-2.55	-5.23	-12.69
Cyp51	3.16	4.71	2.20	2.13	4.74
D15Wsu75e	-3.66	-2.19	-1.94	-4.51	-3.50
Dhrs8	-4.57	-2.55	-1.21	-1.69	-4.90
Dusp2	2.32	2.42	3.92	4.49	2.37
Edem1	-2.07	-2.57	-1.44	-2.54	-5.55
Egr2	13.77	2.93	4.90	3.68	2.65
Ets2	-4.49	-2.63	-2.53	-2.47	-3.43
G7e	3.90	7.77	5.05	3.04	4.04
Gart	2.48	2.66	1.12	1.53	3.66
Gnl3	2.31	2.30	1.65	6.74	7.39
Hsd17b12	3.67	3.44	1.29	1.90	4.02
Hspa5	3.61	3.35	3.91	4.80	4.94
Hspa9a	2.73	2.05	1.17	2.98	4.95
Ian1 (gimap4)	15.76	4.06	5.69	8.97	10.79
Ide	2.07	2.50	1.00	1.29	4.01
Itm2a	9.71	6.35	12.11	18.01	15.11
Lcp1	5.21	2.63	3.65	1.81	8.69
Lip1	-4.32	-3.66	-1.69	-3.38	-5.38
Mdfic	2.63	3.04	-1.15	-1.09	4.34
Mr1	-2.09	-4.26	-2.66	-3.71	-4.87
Mtm1	2.69	2.67	2.26	2.22	6.17
Ndfip1	2.64	2.78	2.54	4.54	5.03
Ndrg1	7.80	6.91	1.92	8.98	13.24
Nfatc1	9.24	8.42	5.12	11.80	11.68
Nfkb1	4.38	2.42	1.97	8.02	7.63
Nme1	2.10	4.02	1.42	2.56	4.72
Noc4l	2.58	2.07	1.14	1.95	3.94
Nola1	2.53	3.17	1.29	3.38	5.54
Nur77 (Nr4a1)	16.06	11.29	25.56	25.51	24.54
OBF-1 (Pou2af1)	14.84	9.98	2.88	7.55	17.00
Osp94	3.35	2.55	3.18	2.95	3.78
Pdcd1	11.98	12.24	3.48	5.73	5.40
Plxnd1	-4.19	-2.75	-2.77	-4.30	-11.64
Pou6f1	-3.75	-2.18	-1.73	-3.39	-8.42
Pp11r	-4.11	-2.55	-1.87	-6.57	-6.81
Psat1	6.14	6.08	1.95	3.27	6.20
Rag2	-7.71	-6.44	-5.86	-6.16	-6.84
Ranbp5	2.37	2.34	-1.10	2.29	6.89
Rars	2.62	2.29	1.64	2.10	4.12
Rmnd5a	-3.67	-2.29	-2.32	-4.36	-5.18
Rrs1	2.81	2.25	1.54	5.23	9.20
Ssbp2	-5.14	-3.72	-1.55	-1.98	-5.47
St13	4.15	3.13	2.60	1.60	4.05
Syncrip	2.66	2.85	1.33	2.65	4.71
Tfrc	2.92	3.08	5.69	2.35	14.73
Timm8a	2.04	2.63	1.42	4.46	3.53
Tiparp	-4.41	-6.72	-3.22	-4.35	-5.45
Traf3	3.02	2.41	1.62	5.22	4.30
Wdr73	-5.49	-3.67	-1.83	-3.19	-5.22
Xrcc6	-3.36	-2.87	-4.79	-4.03	-4.18
Ypel3	-3.24	-2.41	-1.87	-2.17	-3.97
Znrf1	-2.62	-2.40	-2.79	-3.80	-5.31

FIGURE 4. Comparison of negative-selection gene array data sets. *A* and *B*, The data sets generated by Liston et al. (*A*) or Schmitz et al. (*B*) were compared with our data set (388) and those genes common to our list and the other list are shown. Highlighted (gray) are those genes that displayed a similar change when comparing nonselection to negative selection.

below) perhaps suggests that these molecules are the key regulators of thymic clonal deletion (see below). Neither *ian1* nor *lip1* was identified as being regulated by negative selection in any of the other three studies. In fact, Ian1$^{-/-}$ mice displayed normal thymic development, although negative selection in a TCR Tg model was not tested (34). Because Ian1 expression is high in CD4 and CD8SP cells, it appears that expression of Ian1 is not exclusively proapoptotic (34, 35). However, when overexpressed in reaggregation thymic organ culture, Ian1 was demonstrated to induce apoptosis specifically in DP thymocytes regardless of Ag specificity (35). Additionally, it was found to associate with the proapoptotic molecule Bax in vitro (35). Therefore, Ian1 may have a unique proapoptotic role in DP thymocytes. It is unclear what role Lip1, a lysosomal acid lipase, may play in clonal deletion.

Verification of other genes regulated during clonal deletion

Because a large number of genes were identified as being regulated by negative selection, we attempted to verify some of the changes at either the protein or mRNA level. A number of genes regulated by the strength of TCR stimulation were induced by negative selection including CD69, CD5, and CD2 (Fig. 6*A*). B and T lymphocyte attenuator, a molecule that is expressed following positive selection (36), is also induced to a greater extent in negative selection and may represent another molecule whose expression is

A

	Baldwin		Liston		Schmitz		
common name	neg vs non	neg vs pos	neg vs non	neg vs pos	30' neg vs non	60' neg vs non	120' neg vs non
Bim (Bcl2l11)	5.67	3.92	6.49	2.68	13.38	6.65	12.19
Ian1 (gimap4)	15.76	4.06	2.93	-2.38	5.69	8.97	10.79
Lip1	-4.32	-3.66	-2.89	-2.08	-1.69	-3.38	-5.38
Nur77 (Nr4a1)	16.06	11.29	10.17	2.09	25.56	25.51	24.54

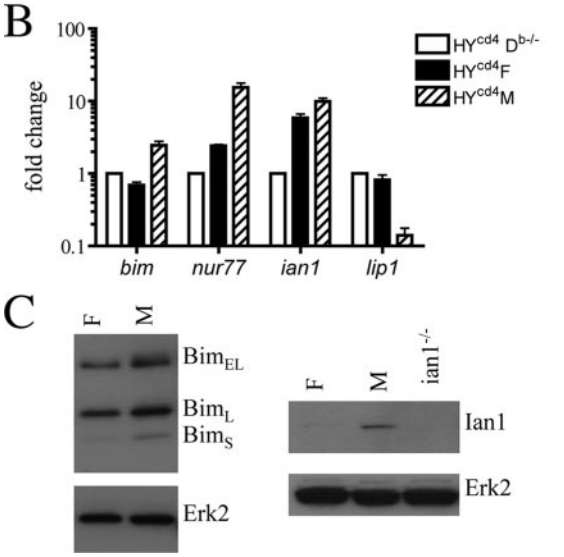

FIGURE 5. Verification of common gene expression changes during T cell development. *A*, Gene changes found in common between the Liston data set, the Schmitz data set, and our data set (388) are depicted. *B* and *C*, Verification of the gene expression changes by qRT-PCR (*B*) or Western blot (*C*) for the common genes. Error bars indicate SD. Western blot analysis is representative of three independent experiments.

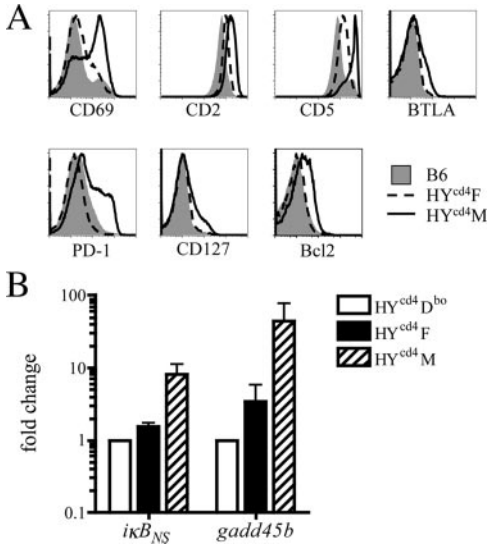

FIGURE 6. Verification of gene expression changes during selection. *A*, DP thymocytes from B6 (shaded histogram), $HY^{cd4}F$ (broken line), and $HY^{cd4}M$ (solid line) were stained with Abs for the indicated proteins and analyzed by flow cytometry. *B*, qRT-PCR on cDNA from sorted DP thymocytes was performed for the indicated genes. Depicted is the fold change relative to nonselecting with *β-actin* as a reference gene. Error bars indicate SD.

regulated by TCR signal strength (Fig. 6*A*). A number of genes involved in cell survival and apoptosis were induced by negative selection. PD-1 was induced on a subset of cells undergoing clonal deletion, but was not induced by positive selection (Fig. 6*A*). PD-1 was also found to be induced by Schmitz and Zucchelli, and fell just below the 2× cutoff comparing negative and nonselection in the Liston data set. Therefore, PD-1 may belong with Bim and Nur77 as a molecule consistently induced during clonal deletion. The role of PD-1 in negative selection is controversial. 2C PD-$1^{-/-}$ animals had a decrease in 2C DP thymocytes suggesting enhanced deletion of 2C (37), whereas PD-1 Tg or PD-$L1^{-/-}$ HY male mice showed no phenotypic difference compared with the wild-type HY counterparts (38). PD-1 was demonstrated to be increased on OT-I thymocytes following positive selection (39), but was not induced in HY^{cd4} female DP thymocytes. Because the positive selection signal from the HY TCR may not be as strong as in OT-I, PD-1 may again represent a protein whose induction is related to TCR signal strength. Somewhat surprisingly to us, molecules involved in the survival of thymocytes and peripheral T cells were also induced by negative selection. CD127 (IL-7Rα) was induced on a subset of thymocytes undergoing clonal deletion (Fig. 6*A*). Additionally, Bcl2 was also up-regulated by negative selection (Fig. 6*A*). It is unclear why these two prosurvival molecules are induced by negative selection, but they may again represent proteins that are expressed in response to TCR ligation regardless of fate. Because clonal deletion is dominant, the expression of these prosurvival molecules may be irrelevant in the face of proapoptotic gene expression.

We also wanted to verify the induction of two other genes, $i\kappa B_{NS}$ and *gadd45b*. $I\kappa B_{NS}$ was cloned by Fiorini et al. (40) as a gene induced by negative selection in the N15 TCR Tg model. It is an inhibitor of NF-κB and, when overexpressed in reaggregation thymic organ culture, enhanced negative selection to anti-CD3 stimulation (40). We found $i\kappa B_{NS}$ was induced in HY^{cd4} male DP thymocytes and this increase was verified by qRT-PCR (Fig. 6*B*). The Schmitz study was the only other one to report this gene as being induced by clonal deletion. *Gadd45β* is a gene that was identified as being highly induced during negative selection in all studies except Huang et al. It did not appear on our gene list because it was lost in the last filter parameter (difference in raw signal values, >300). Using qRT-PCR, we found that *gadd45β* was highly induced during negative selection in the HY^{cd4} model (Fig. 6*B*). Because it was found in all the other gene array studies, it becomes another one of the very few genes in common with most of the data sets (*bim*, *nur77*, and *pd-1* being the others). *Gadd45β* is a gene induced following "stress" stimulus (UV, genotoxic, etc). It has been reported to have both a pro- and antiapoptotic function depending upon the cell type and stress condition (41, 42). Although Gadd45$\beta^{-/-}$ mice have a normal thymic profile (43), they can be induced to develop autoimmunity upon immunization (44). Additionally, Gadd45$\beta^{-/-}$Gadd45$\alpha^{-/-}$ mice develop spontaneous autoimmunity (44). Gadd45β appears to influence the duration and magnitude of Erk and p38 but not Jnk activation (43), which is interesting given the proposed role for differential MAPK signaling in selection (3).

Discussion

Bim and Nur77 as key regulators of clonal deletion

Collectively, the data presented above and compiled from a number of studies examining genes required for thymocyte clonal deletion point to Bim and Nur77 as being critical players in this process. How these two proteins are regulated during clonal deletion is still uncertain. Clearly, Bim is required for thymocytes to undergo negative selection (12), but what is less clear is whether transcriptional and translational induction are necessary. Whereas Crabtree and colleagues (13) demonstrated that transcriptional and subsequent translational induction of Bim was required for anti-CD3/CD28-mediated negative selection in vitro, Bunin et al. (15)

found no evidence of translational induction of Bim in anti-CD3-mediated negative selection in vitro or in vivo. However, Bunin et al. (15) reported that BimEL was phosphorylated both in resting thymocytes and in response to anti-CD3 in vitro and in vivo. The physiological role of Bim phosphorylation in clonal deletion is currently unclear, but in other systems, phosphorylation of Bim can either positively or negatively regulate its proapoptotic function (45, 46). Additionally, only the BimEL and BimL forms appear to be regulated by phosphorylation (47). The phosphorylation of Bim has been reported to alter the subcellular localization of Bim (47), although Bunin et al. found no change in the subcellular localization of Bim following anti-CD3 stimulation of DP thymocytes in vitro or in vivo. It would be of interest to examine the role of the different isoforms of Bim in clonal deletion separately as well as monitoring the effect of single and combined phosphorylation mutants in an in vivo model of clonal deletion.

Nur77 is a steroid orphan nuclear receptor whose induction has long been linked to clonal deletion. As stated previously, it represents a gene that is consistently identified as being up-regulated during negative selection. In support of its role in negative selection, overexpression of full-length Nur77 induced apoptosis in DP thymocytes, whereas overexpression of a dominant-negative Nur77, which likely also affects other Nur77 family members, inhibited Ag-specific clonal deletion (16). Although Nur77-deficient mice showed no obvious phenotype in the thymus, other family members, including Nor1, are thought to be able to compensate for the loss of Nur77 (17). Analysis of $Nur77^{-/-}Nor1^{-/-}$ mice could verify this possibility. In addition to being induced by high-affinity Ag stimulation in DP thymocytes and causing death, peripheral T cells also up-regulate Nur77 in response to high-affinity Ag, but do not undergo apoptosis. A number of reasons for this difference can be envisioned, including differential subcellular localization or posttranslation modification. Recently, these two possibilities were examined by Cunningham et al. (48), and it was observed that, in peripheral T cells upon stimulation, Nur77 was phosphorylated by MAPK and AKT and exported from the nucleus to the cytoplasm, whereas in DP thymocytes, this phosphorylation was dramatically reduced and Nur77 remained nuclear. In support of nuclear localization being proapoptotic, constructs of Nur77 that lack the transactivation domain or are impaired in transactivation activity did not promote apoptosis (18). However, it is unclear whether or not the DNA binding domain is important for the proapoptotic function of Nur77, although some genes demonstrated to be induced by Nur77 (*PD-1* and *IL-7Rα* (49)) were found on our gene list, whereas most others (*Ndr1*, *Ndr2*, etc. (49)) were not found on our or any of the other data sets.

In conclusion, examination of the gene expression changes underlying negative selection has provided a framework from which to dissect this critical biological process. A comparison of all the published data sets identified a small number of common molecules that might constitute key regulators of clonal deletion including Bim, Nur77, PD-1, and Gadd45β. It is of interest that so few common genes were identified in the cross-system analysis. Although this could be due to differences in the method of data analysis, we feel it more likely reflects differences in the model systems studied. Ultimately, there may not be a common mechanism underlying clonal deletion occurring at different stages. Therefore, it may be advantageous to focus on one particular model system and identify functional networks of genes regulated during negative selection. The use of proteomics technologies could facilitate this process by identifying differential protein expression and posttranslational modifications that occur as a result of a high-affinity Ag encounter in the thymus.

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Disclosures

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