Supplemental Materials and Methods

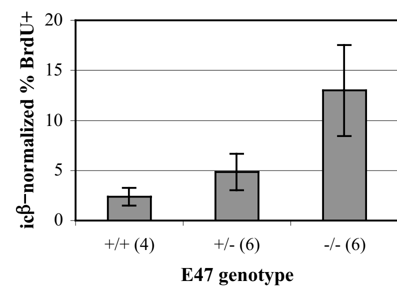
Simultaneous analysis of icβ expression and BrdU incorporation

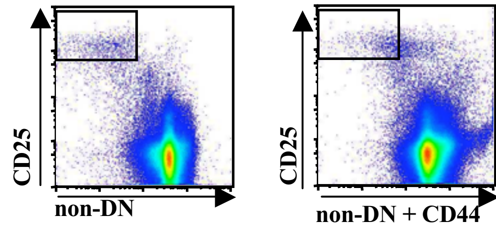
Simultaneous analysis of icβ expression and BrdU incorporation in DN thymocytes required analysis of five parameters. In order to accomplish this using a FACScalibur, which has four fluorescence channels detectors, cells were surface stained with biotinylated antibody conjugates specific for mouse CD4, CD8a, CD3ε, TCRβ, TCRγδ, TER-119, B220, CD11b, GR-1, and NK1.1, followed by streptavidin-peridinin chlorophyll protein (PerCP) and anti-CD25 (clone PC61)-phycoerythrin (PE). Surface-stained cells were fixed, permeabilized and stained with anti-TCRβ-APC as described in Materials and Methods, and then processed as described for detection of BrdU, but staining with anti-CD44-PE-Cy5 together with anti-BrdU. Staining with anti-CD44 was performed together with anti-BrdU because the Dnase treatment required for BrdU detection was found to degrade PE-Cy5 (data not shown). DN3 and DN4 cells were defined as shown in Figures 3 and 4. Separate control stainings were also performed using the same combination of antibodies and procedures, but without anti-CD44, as well as by staining for CD25, CD44 and non-DN markers in separate fluorescence channels and omitting analysis of either BrdU or icβ. The numbers of cells within the defined DN3 and DN4 gates as determined by the approach used for simultaneous icβ and BrdU detection was found to be similar to that determined by the more conventional approach (data not shown).

Supplemental Figure Legends

Figure S1. Normalization of the DN3 BrdU incorporation data presented above with respect to expression of intracellular TCR β chain (icβ). Thymocytes from the same mice analyzed for Figure 3A were separately stained for icβ together with surface markers to define the DN3 subset. The fraction of $ic\beta^{+}$ DN3 cells was determined for each sample, and the mean DN3 $\%ic\beta^{+}$ for each experiment was calculated. The percentage of BrdU-incorporating DN3 thymocytes from each mouse was then normalized as follows: $\%BrdU^{+}$ x $\%ic\beta^{+}$/mean $\%ic\beta^{+}$.

Figure S2. Flow cytometric analysis strategy used to define DN3 thymocytes analyzed in Figure 3B. Left pseudo-color plot: E47+/- thymocytes stained with the combination of anti-CD25-PE and a mix of biotinylated antibodies used to define non-DN thymocytes (described in Materials and Methods) plus streptavidin-PerCP. Right pseudo-color plot: the same thymocytes stained as in the left plot together with anti-CD44-PE-Cy5, which was detected in the same fluorescence channel as PerCp. The shift of the $CD25^{high}$ population in the right plot indicates staining with CD44. The box drawn in each plot depicts an identical gate that defines either all of the $CD25^{high}$ DN thymocytes (left plot), or the $CD25^{high}$, $CD44^{low}$ DN3 subset (right plot).


icβ−normalized % BrdU+
20
15
10
5
0
+/+ (4)
+/- (6)
-/- (6)
E47 genotype


CD25
non-DN
CD25
non-DN + CD44