An influence of CD5 on the selection of CD4-lineage T cells

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Combining CD5-null, MHC-deficient and lineage-specific reporter animals, we have investigated the influence of CD5 on positive selection and the choice of CD4- versus CD8-lineage commitment on broad populations of thymocytes. CD5 has no obvious quantitative effect in wild-type mice. In mice lacking MHC class II molecules, however, increased numbers of transitional, class I-selected $CD4^+ CD8^{int} CD3^{hi}$ cells were positively selected in the absence of CD5. Importantly, they were committed to the CD4 lineage. Our results indicate that CD5 negatively regulates the differentiation of CD4-committed cells in suboptimal conditions, thus perhaps serving to tighten the correlation between restriction of the TCR and lineage choice.

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1 Introduction

Positive selection, or enrichment for cells that can recognize self-MHC molecules, is crucial for the generation of a useful repertoire of T lymphocytes. This highly regulated process begins at the immature $CD4^+ CD8^+$ double-positive (DP) stage of thymocyte differentiation when the majority of cells express low levels of $\alpha\beta$ TCR. Concomitant with positive selection, DP thymocytes are induced to differentiate into $CD4^+ CD8^-$ or $CD4^- CD8^+$ single positive (SP) cells [1, 2]. The key to both positive selection and lineage commitment is recognition by the TCR of MHC molecules expressed on thymic stromal cells.

Much is now known about the pathways by which CD4- and CD8-SP cells differentiate. All thymocytes appear to transiently down-regulate both coreceptors upon positive selection [3]. CD4-committed cells then differentiate in a seemingly straightforward manner – up-regulation of surface CD4 and TCR levels [3–7]. CD8-committed thymocytes, however, appear to differentiate in at least two ways: directly, through up-regulation of surface CD8 (and up-regulation of TCR); or indirectly, through a transitional $CD4^+ CD8^{int} TCR^{hi}$ phenotype, resembling the early stages of CD4-SP cell differentiation, before terminally down-regulating CD4 and up-regulating CD8 [3–6]. Less is known about the signals that underlie these differentiation events.

Aside from the TCR/CD3 complex, several cell-surface molecules are up-regulated on DP thymocytes following positive selection [8]. One is CD5, expressed at low levels on immature $CD4^+ CD8^+$ thymocytes but up-regulated to high levels in both CD4- and CD8-committed cells upon selection [9–11]. CD5 is part of a receptor-signaling complex composed of the CD3ζ chain and protein tyrosine kinases [12–14]. Stimulation through the TCR results in rapid phosphorylation of tyrosine residues within CD5's cytoplasmic domain [15]. Looking at TCR-mediated proliferation of CD4- and CD8-SP thymocytes from CD5-deficient ($CD5^0$) mice, Tarakhovsky et al. [16] have shown that greater responses can be elicited from $CD5^0$ than from wild-type (WT) cells, suggesting that CD5 negatively regulates responses through the TCR. Likewise, when $CD5^0$ mice expressing a class I-restricted TCR transgene were analyzed, hyper-responsive phenomena were observed during thymocyte selection: depending on the apparent affinity of the transgene-encoded TCR, the resulting effect was enhanced positive selection, or a switch from positive to negative selection [16]. These results suggested that CD5 operates during positive selection by

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Abbreviations: $CD5^0$: CD5-deficient **βgal:** β-Galactosidase **II^0:** MHC class II-negative **I^0:** MHC class I-negative **DP:** Double positive **SP:** Single positive **FDG** fluorescein digalactopyransoide

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dampening the responsiveness of DP cells to TCR-mediated signals. The previous study [16] focused on just a few TCR, all class I-restricted. Thus, it seemed necessary to evaluate its conclusions with a broad population of thymocytes and with class II-restricted cells, in particular. We have done this by crossing CD5-null, MHC-deficient and lineage-specific reporter mice together, permitting us to assay bulk populations of CD4- and CD8-SP thymocytes.

2 Results

2.1 Transitional MHC class I-reactive, $CD4^+ CD8^{int} CD3^{hi}$ thymocytes are increased in the absence of CD5

To determine how CD5 influences positive selection of heterogeneous populations of T cells, we crossed $CD5^0$ mice with MHC class II-negative (II^0) animals, and analyzed thymocyte populations by three-color flow cytometry, using antibodies specific for CD4, CD8 and CD3. Thymocyte compartments in mice lacking only CD5 were not overtly different from those of WT animals (Fig. 1A, top panels), as previously described [17]. However, when $CD5^0 II^0$ double-deficient mice were compared with II^0 single-deficient animals, there was a consistent (average 1.8-fold) increase in the number of $CD4^+ CD8^{int} CD3^{hi}$ cells in the former (Fig. 1A, middle panels; Fig. 1B). These cells were previously shown to be selected on MHC class I molecules [18]. The transitional cells in $CD5^0 II^0$ animals appeared more differentiated than the same population from II^0 mice in that CD8 expression was on average more down-regulated (Fig. 1A). However, the expression of other maturation markers was similar in the two cases, there being high levels of CD24, CD69 and peanut agglutinin (PNA)-binding polysaccharides (not shown). In addition, the percentage of $NK1.1^+ CD4^+$ cells within the $CD4^+ CD8^{int}$ populations of $CD5^0 II^0$ and II^0 mice was nearly identical (not shown). Despite the increased number of transitional intermediates in double-deficient mice, there were not significantly more fully mature $CD4^+ CD8^-$ thymocytes. Since the $CD4^+ CD8^{int}$ population is composed of both CD4- and CD8-lineage cells [4, 5], we also wondered whether more CD8-lineage cells were generated in $CD5^0 II^0$ than in II^0 animals. However, both the $CD4^{int} CD8^+ CD3^{hi}$ and the mature $CD4^- CD8^+ CD3^{hi}$ populations were the same in both types of mice.

Similarly, $CD5^0$ mice were crossed with I^0 animals to determine whether CD5 influences the selection of MHC class II-reactive thymocytes. The absence of CD5 did not detectably alter the thymocyte compartments in class I-deficient mice (Fig. 1A, bottom panels).

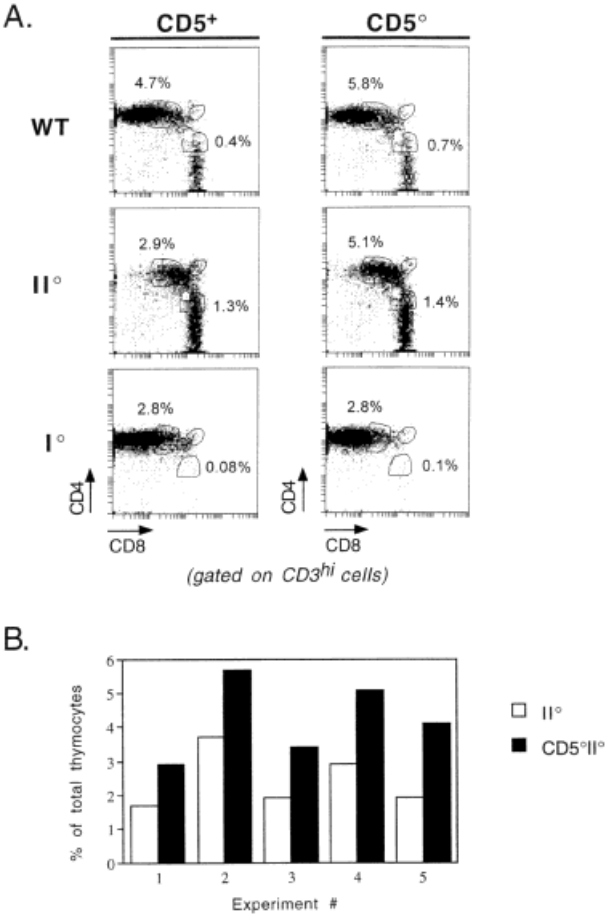

Figure 1. Increased transitional $CD4^+ CD8^{int} CD3^{hi}$ thymocytes in $CD5^0 II^0$ mice. (A) $CD3^{hi}$ thymocytes from WT, $CD5^0$, II^0, $CD5^0 II^0$, I^0, and $CD5^0 I^0$ mice were gated according to their CD4/CD8 expression into distinct populations: transitional $CD4^+ CD8^{int}$ cells, DP cells, intermediate $CD4^{int} CD8^{lo}$ cells. Percentages are of total thymocytes for this particular experiment. (B) Results from five experiments comparing the percentages of $CD4^+ CD8^{int} CD3^{hi}$ thymocytes in II^0 versus $CD5^0 II^0$ animals.

Interestingly, CD3 levels on $CD4^+ CD8^{int}$ thymocytes were affected by the CD5-null mutation. $CD69^+$ thymocytes (cells which had recently undergone positive selection) from $CD5^0$ mice expressed markedly lower CD3 levels compared with those from WT animals (Fig. 2). This result is intriguing in light of recent data showing that CD5 expression is proportional to the level and signaling capacity of the TCR [11], and suggests that CD5 and TCR molecules may be partially dependent on one another for full surface expression.

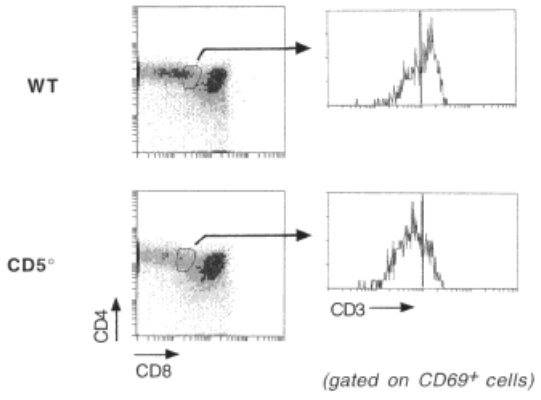

Figure 2. Lower CD3 levels on $CD4^+CD8^{int}CD69^+$ thymocytes of $CD5^0$ mice. $CD4^+CD8^{int}CD69^+$ thymocytes from WT and $CD5^0$ animals were gated and the CD3 expression of these cells are shown in the corresponding histograms.

2.2 CD5 influences CD4/CD8-lineage commitment

Since transitional $CD4^+CD8^{int}$ thymocytes were increased in $CD5^0II^0$ mice, but without a resulting increase in the numbers of CD8-SP thymocytes, we hypothesized that CD5 might preferentially influence the positive selection of class I-reactive, CD4-committed cells. To test this, we took advantage of a recently described "knock-in" mouse line ($CD4^{+/L}$) in which one of the endogenous CD4 alleles was replaced by β-galactosidase (βgal; [5]). By virtue of its expression pattern and the shorter half-life of its protein product, the βgal reporter appears to be a faithful and early indicator of CD4 gene transcription: CD4-committed thymocytes express high levels of βgal, CD8-committed thymocytes only background levels [5].

II^0 and $CD5^0II^0$ mice carrying a $CD4^{+/L}$ genotype were generated and their thymocytes stained with fluorescein digalactopyranoside (FDG) to detect βgal expression, in addition to antibodies against CD4, CD8 and CD3 (Fig. 3). In MHC-expressing $CD4^{+/L}$ animals (left panel), the transitional $CD4^+CD8^{int}CD3^{hi}$ thymocytes were largely $βgal^{hi}$, CD4-committed cells (2.9 ± 0.6 % of total thymocytes), although some $βgal^{lo}$, CD8-committed cells (0.7 ± 0.2 %) were also present, as previously described [5]. In contrast, the $CD4^{int}CD8^+CD3^{hi}$, CD8-committed population expressed background levels of the βgal reporter (0.5 ± 0.2 %). Similar values were obtained in $CD5^0CD4^{+/L}$ mice (not shown). In $II^0CD4^{+/L}$ mice (middle panel), significantly fewer $βgal^{hi}$ cells (1.8 ± 0.1 %) were found in the $CD4^+CD8^{int}CD3^{hi}$ population compared with the WT equivalent, with a proportional increase in the numbers of $βgal^{lo}$ cells (0.9 ± 0.04 %). In agreement with previous findings, about one-third of the $CD4^+CD8^{int}CD3^{hi}$ cells in II^0 mice are committed to the CD8 lineage (Fig. 3; [5]). When thymocytes from $CD5^0II^0CD4^{+/L}$ mice were analyzed (right panel), the increase previously observed in the transitional $CD4^+CD8^{int}CD3^{hi}$ population could be attributed almost exclusively to the $βgal^{hi}$, CD4-committed subset, such that the percentage of $βgal^{hi}$ cells returned to that of WT levels (3.1 ± 0.2 %). This shift was consistently seen: in five $CD5^0II^0CD4^{+/L}$ mice tested, only the $βgal^{hi}$ subset was significantly affected (although slight increases were sometimes observed with the $βgal^{lo}$ subset; Table 1).

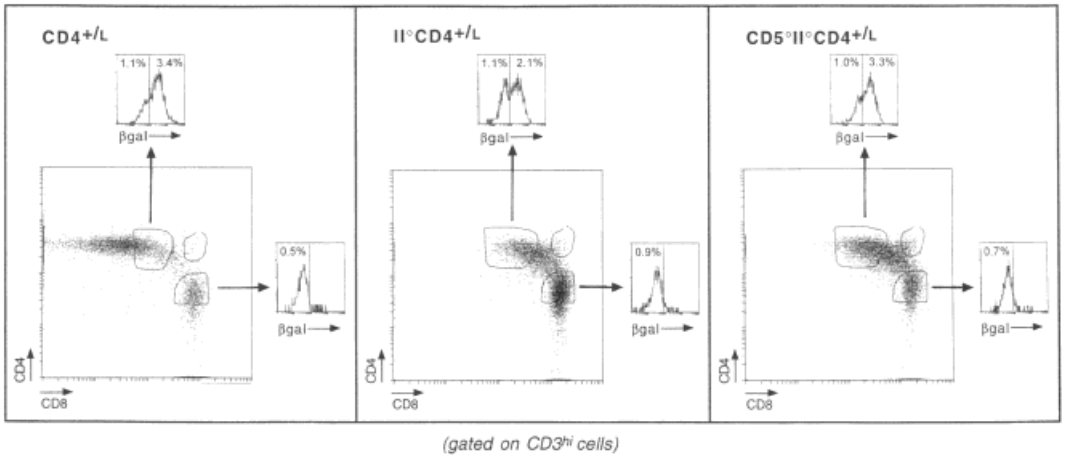

Figure 3. Increased CD4-lineage cells in the absence of CD5. Expression of the βgal reporter in $CD3^{hi}$ thymocyte populations from $CD4^{+/L}$, $II^0CD4^{+/L}$ and $CD5^0II^0CD4^{+/L}$ mice are shown. Populations were gated as in Fig. 1 and the βgal expression of the gated cells are shown in the respective histograms. Percentages of the $βgal^{hi}$ and $βgal^{lo}$ cells are of total thymocytes for this experiment.

Table 1. Comparison of βgal^{hi} versus βgal^{lo} subpopulations in CD4$^+$ CD8^{int} CD3^{hi} thymocytes from CD4$^{+/L}$, Il^0 CD4$^{+/L}$ and CD5^0 Il^0 CD4$^{+/L}$ mice[a)]

	CD4$^+$ CD8^{int} CD3^{hi} thymocytes					
	βgal$^+$			βgal$^-$		
	CD4$^{+/L}$	Il^0 CD4$^{+/L}$	CD5^0 Il^0 CD4$^{+/L}$	CD4$^{+/L}$	Il^0 CD4$^{+/L}$	CD5^0 Il^0 CD4$^{+/L}$
Expt # 1	3.4 3.2 2.9	1.9	**3.3**	1.0 0.9 0.6	0.9	1.1
Expt # 2	1.9	2.1	**3.4** **3.1** **2.8**	0.5	0.9	1.5 1.0 1.1
Expt # 3	3.3	1.5	**2.9**	0.5	1.0	1.2
Mean ± SD	2.9 ± 0.6	1.8 ± 0.1	**3.1 ± 0.2**	0.7 ± 0.2	0.9 ± 0.04	1.2 ± 0.2

a) Percentages of total thymocytes from different mice are shown; five animals for CD4$^{+/L}$, three for Il^0 CD4$^{+/L}$, and five for CD5^0 Il^0 CD4$^{+/L}$. Results for the βgal^{hi}-expressing cells from CD5^0 Il^0 CD4$^{+/L}$ mice are highlighted in bold.

These results suggest that CD5 preferentially hinders commitment of class I-selected cells to the CD4 lineage.

2.3 CD5 does not affect the kinetics of differentiation

Although our results suggested that CD5 affects CD4/CD8-lineage choice upon positive selection, it was possible that the increase in the immature CD4$^+$ CD8^{int} CD3^{hi} βgal^{hi} population in CD5^0 Il^0 CD4$^{+/L}$ animals was due to: (i) an increase in the rate of thymocytes differentiating into the transitional population; (ii) a decrease in the kinetics of cells exiting this population due to enhanced survival and accumulation of class I-reactive, CD4-committed thymocytes; and/or (iii) a slowdown in the differentiation of CD8-committed thymocytes which had not yet down-regulated their βgal expression.

To address these possibilities, we compared the kinetics of generation and disappearance of transitional CD4$^+$ CD8^{int} CD3^{hi} thymocytes from WT, CD5^0, Il^0 and CD5^0 Il^0 mice using the bromodeoxyuridine (BrdU) pulse-labeling technique [19, 20]. By following the proportion of BrdU-labeled cells over time, one can measure the rate of entry and exit for a given population. Mice were injected with BrdU at day 0 and sacrificed 1–6 days later; their thymocytes were stained with antibodies against CD4, CD8, CD3 and BrdU, and analyzed by flow cytometry. The results are presented in Fig. 4. In WT mice (upper left), a significant portion of CD4$^+$ CD8^{int} CD3^{hi} thymocytes were labeled with BrdU by day 2, peaking by day 3 and decreasing by day 4, in agreement with Lucas et al. [7]. In CD5^0 Il^0 mice (lower right), labeled CD4$^+$ CD8^{int} CD3^{hi} cells appeared and then disappeared with the same kinetics as littermate controls. In fact, the labeling kinetics of all thymic populations were unaltered in CD5^0 Il^0 mice (not shown).

Thus, the increase observed in the transitional CD4$^+$ CD8^{int} CD3^{hi} population when CD5 is absent must reflect larger numbers of cells entering this compartment. Our results indicate that more CD4-lineage thymocytes are selected in Il^0 mice in the absence of CD5.

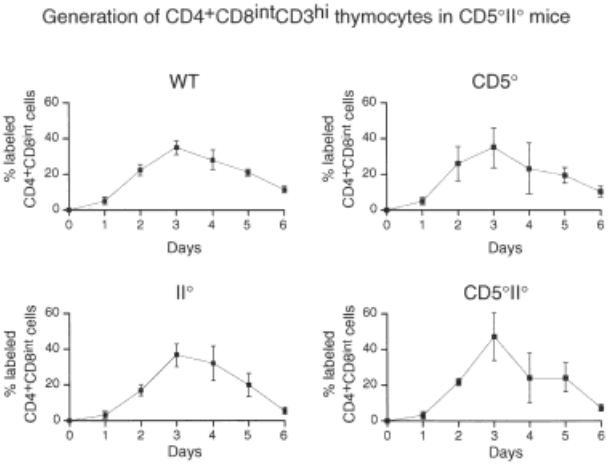

Figure 4. Generation of BrdU-labeled CD4$^+$ CD8^{int} CD3^{hi} thymocytes in WT, CD5^0, Il^0 and CD5^0 Il^0 mice from 1 to 6 days following initial pulse. The % of labeled CD4$^+$ CD8^{int} CD3^{hi} cells at each timepoint was compiled from three mice.

3 Discussion

The CD5 molecule was shown previously to influence TCR-mediated selection processes, as the CD5-null mutation had distinct effects on TCR tg mice expressing different MHC class I-restricted receptors [16]. We have investigated the role of CD5 in the selection of a broad repertoire of thymocytes expressing TCR randomly generated by the rearrangement process, focusing on the transitional intermediates, best visualized in the absence of MHC molecules. In $I\!I^0$ mice also lacking CD5, there was a consistent increase in the number of transitional $CD4^+\,CD8^{int}$ thymocytes (Fig. 1), cells known to be selected on MHC class I molecules [18]. Importantly, this augmentation involved only CD4-lineage cells: in $CD5^0\,I\!I^0\,CD4^{+/L}$ mice, it was largely the $CD4^+\,CD8^{int}$ subset expressing high levels of βgal that was affected. The increase was due to enhanced selection rather than a kinetic effect because there was no change in the rates of appearance or disappearance of cells within the transitional population, as measured by BrdU-tracing experiments. As in $I\!I^0$ mice, the $CD4^+\,CD8^{int}\,CD3^{hi}$ cells did not survive for long in $I\!I^0\,5^0$ thymi, mature CD4-SP thymocytes remaining rare.

CD5's effect on the selection of CD4-lineage cells was intriguingly specific, as there was no analogous augmentation in $CD4^{int}\,CD8^+$ or CD8-committed $CD4^+\,CD8^{int}\,\beta gal^{lo}$ thymocytes. It also appears to be quite general. Greater numbers and activity of CD4-lineage, class II-restricted cells have also been detected in CD4-null mice also lacking CD5 (N. Killeen, manuscript submitted). In addition, we noted that $CD5^0\,I\!I^0$ mice showed an increased number of peripheral $CD4^+$ T lymphocytes compared with $I\!I^0$ mice (not shown). These cells are known to be restricted by non-classical class I molecules like CD1 [21], suggesting that CD5 may dampen the selection or survival of these peculiar CD4-lineage cells as well. Thus, the absence of CD5 enhances CD4-lineage differentiation in three separate contexts.

Given the known effects of CD5 on signal transduction through the TCR [16], the most straightforward interpretation of the present results is that the absence of CD5 lowers the threshold of positive selection, as suggested previously [16]. Interestingly, commitment to the CD4 lineage is favored under these conditions. A similar increase is not observed in mice expressing class II molecules, however, but one could argue that the number of mature CD4-lineage cells is already at its maximum in these animals, and that competition for selection niches is the rate-limiting factor, not the ability of individual TCR to induce selection – several results compatible with such a competition environment have been reported [22–24]. Thus, one may only be able to observe the effect of the CD5 deficiency in these three peculiar, non-saturating conditions.

However, a more complex interpretation could also be proposed. In all three cases where the absence of CD5 enhances the differentiation of CD4-lineage cells, TCR signaling was disconnected from CD4 coreceptor signaling: because CD4 was absent, because the MHC class II ligand was absent, or because the non-classical MHC ligand could not engage CD4. Along this line of reasoning, only the suboptimal signals elicited through the TCR without coreceptor co-engagement would be boosted in the absence of CD5. Thus, CD5 may serve to dampen the noise of unproductive TCR/MHC engagements to which T cells must constantly be subjected, without affecting the *bona fide* signals derived from stable TCR/CD4 co-engagement of agonistic MHC/peptide ligands.

These observations are certainly consistent with the idea that CD4/CD8-lineage choice is influenced by the affinity/avidity of the TCR for positively selecting ligands on stromal cells [25–30]. According to this quantitative model, stronger signals may drive a DP thymocyte preferentially along the $CD4^+$ or the $CD8^+$ pathway, although it remains unclear at present which lineage is best promoted by a strong interaction. In this regard, if the effect of CD5 is to depress TCR-mediated signaling, then our finding that commitment to the CD4 lineage is favored in the absence of CD5 supports the notion that a stronger signal drives CD4 commitment [27].

4 Materials and methods

4.1 Antibodies and cytofluorimetric analyses

The following antibodies were used: FITC-labeled anti-CD8α, biotinylated anti-CD8α, PE-labeled anti-CD4 (Caltag Laboratories, Inc., Burlingame, CA); Red613-labeled anti-CD8α (Gibco BRL); FITC-labeled anti-BrdU (Becton Dickinson Immunocytometry Systems, San Jose, CA); KT3, specific for CD3 [31]. Texas Red-, Cy5- and AMCA-conjugated anti-rat antibodies, and streptavidin-Cy5 and -Texas Red, were purchased from Jackson Immunoresearch Laboratories (West Grove, PA) or Caltag Laboratories. Staining of thymocyte suspensions were performed as described [32].

Three- or four-color flow cytometry analyses were performed on a Coulter Elite cytometer equipped with 4-decade logarithmic amplifiers. List-mode data were collected on 5×10^4 or 10^5 cells.

4.2 FDG staining

FDG staining was adapted from Nolan et al. [33]. Briefly, 4 × 10^6 6 × 10^6 cells were first stained, then washed and resuspended in 120 μl PBS. FDG (Molecular Probes, Inc. or Sigma Chemical Co.) at the stock concentration of 100 mM in H_2O/DMSO/ethanol, 1:1:1 (v/v), was diluted to a working condition of 7.5 mM with H_2O. Both cells and diluted FDG were warmed to 37 °C for 5 min. Thereafter 80 μl of warmed FDG were added to cells while vortexing. Cells were incubated for 5 min at 37 °C. FDG loading was stopped by adding 2 ml of ice-cold PBS. Cells were kept on ice for 5 min, then transferred to a 15 °C H_2O bath for 15–30 min to enhance βgal activity. Cells were then ready to analyze, or were kept on ice until analysis by flow cytometry.

4.3 BrdU incorporation and analysis

Mice were injected twice intraperitoneally with 1 mg BrdU 4 h apart, as described [19], then killed at the indicated timepoints following the initial injection. Thymocytes were stained for the expression of various surface antigens, as described above. BrdU incorporation was detected as described by Tough and Sprent [20]. Briefly, surface-stained cells were fixed for 30 min in 70 % ethanol on ice, and overnight at 4 °C in 1 % paraformaldehyde, 0.01 % Tween 20. Cells were washed with PBS and resuspended in 150 mM NaCl, 5 mM $MgCl_2$, 10 μM HCl containing 300 μg/ml DNase I (Sigma Chemical Co.) for 10 min at room temperature. Washed cells were then incubated with FITC-conjugated anti-BrdU for 30 min at room temperature, then analyzed as above.

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