CD45RA and CD45RB\textsuperscript{high} Expression Induced by Thymic Selection Events

By Valerie A. Wallace, Wai Ping Fung-Leung,\textsuperscript{*} Emma Timms, Dawn Gray, Kenji Kishihara, Dennis Y. Loh,\textsuperscript{†} Josef Penninger, and Tak W. Mak

From the Ontario Cancer Institute, Department of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario M4X 1K9; the \textsuperscript{*}R. W. Johnson Pharmaceutical Research Institute, Don Mills, Ontario, M3C 1L9, Canada; and the \textsuperscript{†}Departments of Medicine, Genetics, Molecular Microbiology, and Immunology, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110

Summary

CD45 is a protein tyrosine phosphatase involved in T and B cell signaling. While peripheral T cells switch CD45 isoforms upon activation, events leading to exon switching during T cell development in the thymus have not been determined. The expression of high molecular weight isoforms of CD45 was examined on thymocytes from nontransgenic and T cell receptor (TCR) transgenic mice. All thymocytes from nontransgenic mice were CD45RB\textsuperscript{*} as assessed by staining with MB23G2, an anti-CD45RB\textsuperscript{*} monoclonal antibody. Interestingly, there was a small population (1–3%) of thymocytes that displayed a higher intensity of staining with MB23G2, CD45RB\textsuperscript{high}. CD45RB\textsuperscript{high} thymocytes were found in all subsets defined by CD4 and CD8 expression and were also present within the TCR-\textalpha/-\textbeta\textsuperscript{high} population. To analyze whether or not CD45 expression correlated with thymic selection events, expression of CD45RB\textsuperscript{high} and a second isoform, CD45RA, was examined on thymocytes from H-Y and 2C TCR transgenic mice and found to correlate with positive and negative selection events but did not occur in nonselecting backgrounds. CD45RA and CD45RB\textsuperscript{high} upregulation was also not observed in transgenic mice backcrossed into CD8-deficient mice, a scenario in which there is no positive selection of transgene-expressing thymocytes. These data suggest that modulation of CD45 isoform expression may be involved in thymic selection events.

CD45 is a glycosylated transmembrane protein tyrosine phosphatase expressed on all hemopoietic cells except erythrocytes and platelets (reviewed in reference 1). Multiple isoforms of CD45 are generated through differential splicing of extracellular domain exons 4, 5, 6, and 7 (1, 2). The low molecular weight isoform of CD45, which lacks exons 4–6, is referred to as CD45RO; however, in the murine system there are no antibodies available that detect this isoform (1). CD45 isoforms reactive with anti-exon 4-, 5-, and 6-specific mAbs are referred to as CD45RA, CD45RB, and CD45RC, respectively (1). In both human and murine systems, differential isoform expression is cell type, differentiation, and activation stage specific (1). Cell surface expression of CD45 is required for antigen receptor-mediated signaling in T and B cells (3–5). A B cell surface molecule, CD22, has been reported to bind the lowest molecular weight isoform of human CD45, CD45RO (6). However, the functions and ligands for other CD45 isoforms still remain speculative.

T cells mature in the thymus, where they are subjected to selection processes resulting in the production of a peripheral T cell pool that is restricted to self-MHC molecules but is self-tolerant (7). Studies with TCR V\textbeta-specific antibodies and TCR transgenic mice have provided direct evidence for tolerance induction by clonal deletion of T cells reactive against self-antigens and positive selection of TCR-\textalpha/\textbeta by MHC molecules (7–9). The results of these and other studies have established a model of thymic differentiation in which TCR/CD3\textsuperscript{−} CD4\textsuperscript{−} CD8\textsuperscript{−} (double-negative; DN\textsuperscript{1}) thymocyte precursors give rise to immature CD4\textsuperscript{+} CD8\textsuperscript{−} (double-positive; DP) thymocytes expressing low levels of TCR/CD3. Thymocytes that are positively selected through TCR interaction with self-MHC mature into CD4\textsuperscript{+} CD8\textsuperscript{−} or CD4\textsuperscript{−} CD8\textsuperscript{+} single-positive (SP) cells expressing high levels of TCR/CD3 (9, 10). Positive and negative selection most likely involves signaling through the TCR. However, the molecular events associated with these signals are not well understood.

\textsuperscript{1}Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive.
Activation of peripheral T cells induces changes in alternative splicing of the CD45 mRNA, which result in changes of CD45 isoform expression at the cell surface (11). However, it is not known if signaling through the TCR during thymic development can also induce changes in CD45 isoform expression. Thus, CD45 isoform expression was examined on thymocytes from nontransgenic and TCR transgenic animals. Previous studies have indicated that the majority of immature murine thymocytes express low molecular weight CD45 on the cell surface (12). In contrast, mature CD4 SP and CD8 SP thymocytes were shown to express larger molecular weight CD45 glycoproteins, suggesting that there is a molecular switch in regulation of CD45 protein expression upon maturation of thymocytes to the SP lineage (12). We present evidence that the majority of thymocytes are also CD45RB+ when stained with MB23G2, an anti-CD45RB-specific mAb (13). Two populations of CD45RB+ thymocytes were observed, CD45RBlow and a small population (1–3%) with CD45RBhigh expression. CD45RBhigh thymocytes were present in all four subsets defined by CD4 and CD8 expression and in a population of TCR-α/βhigh thymocytes. Expression of CD45RBhigh and CD45RA on TCR transgenic–positive thymocytes from two different TCR transgenic mouse models was also investigated. Expression of CD45RBhigh and CD45RA on transgene-positive thymocytes was found to correlate with selection events. These results suggest that modulation of CD45 isoform expression may be involved in thymic selection events.

Materials and Methods

Mice. 6-wk-old female BALB/c, C57BL/6, and DBA/2 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CD8-deficient mice, and H-Y and 2C transgenic lines, have been described (9, 14, 15) and were bred in our own facilities. H-Y and 2C transgenic mice expressing T3.70 are blocked at the DN stage (9). CD45RBhig h expression was observed in a population of H-2b/b thymocytes from female H-2 b/b transgenic mice. However, in contrast to previous reports suggesting that the majority of immature thymocytes express the lowest molecular weight isoform of CD45, which lacks exons 4, 5, and 6 (12). Moreover, there was a small population (1–3%) of thymocytes that had a higher intensity of CD45RB staining, hereafter referred to as CD45RBhigh (Fig. 1). Repeat analysis yielded consistent results. Triple staining for CD45RB, CD4, and CD8 expression on thymocytes revealed that CD45RBhigh cells were present in all thymic CD4/CD8 subsets and were enriched in the DN and CD8 SP populations (Table 1). CD45RBhigh cells were also present on a population of TCR-α/βhigh thymocytes (Fig. 1). Similar results were obtained with two other CD45RB-specific antibodies, C363.16A and MB4B4 (16, 18, data not shown).

The observation that CD45RBhigh expression was most apparent among TCR-α/βhigh thymocytes suggested that expression of CD45RBhigh might be a marker associated with the maturation of this subset. TCR transgenic mice provide a good model system in which to test this hypothesis since the differentiation of transgenic thymocytes can be followed with mAbs specific for the transgenic TCR. Therefore, thymocytes from two TCR transgenic models, H-Y and 2C, were analyzed for CD45RBhigh expression in positive, neutral (nonscoring), and negative selection backgrounds.

CD45RA and CD45RBhigh Expression on H-Y Transgenic Thymocytes. The H-Y transgenic TCR is specific for the male H-Y antigen in the context of H-2Db. Positive selection of this transgenic TCR in female H-2b/b mice is characterized by transgene expression, detected with the mAb T3.70 specific for the transgenic α chain, on mature CD8 SP thymocytes (9). When the transgene is expressed in a nonscoring background, H-2d/d, thymocytes expressing T3.70 are blocked at the DP immature stage of development (9). Although there is a small population of thymocytes in H-2d/d mice that express high levels of the transgenic receptor, it has been established that these cells are DN thymocytes (9). CD45RA, CD45RBhigh, and T3.70 expression were examined on thymocytes from female H-2b/b and H-2d/d transgenic mice. CD45RA and CD45RBhigh expression was observed in a population of T3.70high cells in H-2b/b mice but not in H-2d/d mice. As a control for background fluorescence, samples were incubated with second-stage goat anti-rat–conjugated reagents followed by staining with the appropriate anti-TCR mAb. Samples were run on a FACScan® (Becton Dickinson & Co.). Data from viable cells were collected using a live gate by a combination of forward and 90° light scatter, and 18,000 live gated events were collected. CD45RA and CD45RB expression on transgenic thymocytes was determined in two (H-Y transgenic mice) and three (2C transgenic mice) independent experiments. Representative values from one experiment are shown in Figs. 2, 3, and 4.

Results

CD45RB Expression on Thymocytes. Thymocytes from C57BL/6, BALB/c, and DBA/2 mice were stained for CD45RB expression using the CD45 exon 5–specific mAb MB23G2. All thymocytes were found to be CD45RB+ (Fig. 1), which is in contrast to previous reports suggesting that the majority of immature thymocytes express the lowest molecular weight isoform of CD45, which lacks exons 4, 5, and 6 (12). Moreover, there was a small population (1–3%) of thymocytes that had a higher intensity of CD45RB staining, hereafter referred to as CD45RBhigh (Fig. 1). Repeat analysis yielded consistent results. Triple staining for CD45RB, CD4, and CD8 expression on thymocytes revealed that CD45RBhigh cells were present in all thymic CD4/CD8 subsets and were enriched in the DN and CD8 SP populations (Table 1). CD45RBhigh cells were also present on a population of TCR-α/βhigh thymocytes (Fig. 1). Similar results were obtained with two other CD45RB-specific antibodies, C363.16A and MB4B4 (16, 18, data not shown).

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T3.70\textsuperscript{high} thymocytes from nonselecting H-2\textsuperscript{d/d} mice (Fig. 2). Triple staining analysis revealed that in H-2\textsuperscript{b/b} mice the CD45RA\textsuperscript{+} T3.70\textsuperscript{high} thymocytes belonged to the CD8 SP (65%), DP (5%), and DN (30%) subsets. CD45RB\textsuperscript{high} T3.70\textsuperscript{high} cells belonged to the CD8 SP (60%), DP (35%), and DN (5%) subsets. It has been previously demonstrated that T3.70\textsuperscript{high} CD8 SP and T3.70\textsuperscript{high} DP thymocytes arise from positive selection (9, 20). Therefore, in H-2\textsuperscript{b/b} mice, the majority (>70%) of thymocytes with CD45RA and CD45RB\textsuperscript{high} expression have been positively selected. It should be noted that of the total T3.70\textsuperscript{high} CD8 SP population, only 56% were CD45RA\textsuperscript{+} and 52% CD45RB\textsuperscript{high}.

Selection of the H-Y TCR is dependent upon CD8 expression since this TCR cannot be selected when the transgene is backcrossed into mice deficient for CD8 expression (CD8\textsuperscript{-/-}) (15, 20a). T3.70\textsuperscript{+} thymocytes from CD8\textsuperscript{-/-} female H-2\textsuperscript{b/b} transgenic mice did not express CD45RA or CD45RB\textsuperscript{high} (data not shown). Therefore, the development of T3.70\textsuperscript{+} thymocytes expressing CD45RA or CD45RB\textsuperscript{high} is dependent upon positive selection of the transgenic TCR.

Table 1. CD45RB\textsuperscript{high} Expression on Thymic Subsets Defined by CD4 and CD8 Expression

| Subset         | Percent of CD45RB\textsuperscript{high} thymocytes |
|----------------|---------------------------------|
| CD4\textsuperscript{+}CD8\textsuperscript{-} | 21.8 ± 5.1                      |
| CD4\textsuperscript{+}CD8\textsuperscript{+} | 1.0 ± 1.0                       |
| CD4\textsuperscript{+}CD8\textsuperscript{-} | 6.4 ± 1.4                       |
| CD4\textsuperscript{-}CD8\textsuperscript{+} | 14.0 ± 1.0                      |

Thymocytes from BALB/c and DBA/2 mice were triple stained with antibodies against CD4, CD8, and CD45RB. The percentage of CD45RB\textsuperscript{high} cells in each subset was determined by gating on individual subsets at the analysis stage. The results are presented as the mean percent of CD45RB\textsuperscript{high} cells ± SD and represent the data from two individual mice of each mouse strain.
and was not observed in either of two instances in which there was a lack of positive selection: absence of H-2 D^b expression or deficient CD8 expression.

CD45RA and CD45RB_{high} expression was also examined on T3.70^+ thymocytes developing in the presence of the deleting H-Y antigen in male H-2^b/b transgenic mice. Negative selection in male H-Y mice results in a drastic reduction of DP thymocytes (9). The remaining T3.70^+ CD8 SP thymocytes display a CD8_{low} phenotype, and it has been suggested that they represent cells that have escaped negative selection by downregulating CD8 surface expression (9, 21). In male transgenic mice there were populations of CD45RA^{+} T3.70^{+} and CD45RB_{high} T3.70^{+} thymocytes (Fig. 2). Triple staining revealed that CD45RA^{+} T3.70^{+} thymocytes belonged to the CD8_{low} (16%), DN (78%), and the DP (6%) subsets. Similarly, CD45RB_{high} T3.70^{+} thymocytes belonged to CD8_{low} (15%), DN (91%), and DP (4%) subsets. Since the development of the T3.70^{+} CD8_{low} subset is dependent upon the presence of H-Y/H-2 D^b (22), CD45RA and CD45RB_{high} expression was occurring on a selected subset of transgene-positive thymocytes. Within the T3.70^{+} CD8_{low} subset, only 50% and 43% of thymocytes were CD45RA^{+} and CD45RB_{high}, respectively.

Negative selection of the H-Y TCR in male mice is dependent upon CD8 expression since efficient deletion of transgenic thymocytes did not occur in CD8^-/- male transgenic mice (20a). CD45RA expression was not observed on thymocytes from CD8^-/- male mice, however, CD45RB_{high} expression on T3.70^+ cells was detected (data not shown).

**CD45RA and CD45RB_{high} Expression on 2C Transgenic Thymocytes.** CD45RA and CD45RB_{high} expression on thymocytes from H-Y mice correlated with selection events. To confirm these results, thymocytes from 2C TCR transgenic mice were stained for CD45RA, CD45RB_{high}, and transgene expression. The 2C transgenic TCR, detected with the anticonnotypic mAb 1B2, is alloreactive against H-2 L^d (19). Maturation of 1B2^+ T cells to the CD8 SP compartment requires the presence of the H-2 K^d molecule (23, 24). Thymocytes from 2C transgenic mice backcrossed into a nonselecting background, H-2 K^b^a, are blocked at the immature DP stage of development (23). In nonselecting mice, 1B2_{high} thymocytes belong to the DN compartment (25). As shown in Fig. 3, there are populations of CD45RA^{+} 1B2_{high} and CD45RB_{high} 1B2_{high} thymocytes present in H-2 K^b^a mice that are absent in nonselecting H-2 K^b^a mice. Triple staining revealed that CD45RA^{+} 1B2_{high} thymocytes from H-2 K^b^a mice were CD8 SP (82%), DP (10%), and DN (8%); CD45RB_{high} 1B2_{high} thymocytes were found among CD8 SP (70%), DP (16%), and DN (12%) thymocyte subsets. Within the 1B2^+ CD8 SP mature subset in H-2 K^b^a mice, only 20% of thymocytes expressed CD45RA or CD45RB_{high}.

CD45RA^{+} 1B2_{high} thymocytes were also observed in nonselecting CD8^-/- H-2 K^b^a 2C transgenic mice (Fig. 4; 20a). Therefore, the appearance of transgene-positive thymocytes in 2C transgenic mice expressing CD45RA and CD45RB_{high} was dependent upon selection since these populations were not observed when positive selection did not occur due to the absence of H-2 K^b or CD8 expression.

In Fig. 3, top left, there is also a population of CD45RA^{+} 1B2_{high} thymocytes (1.0%) with higher intensity CD45RA expression than CD45RA^{+} 1B2_{high} thymocytes. These cells were identified as B cells by their IgM^+, Thy-1^-, and TCR-a/b^+ surface phenotype (data not shown).

Negative selection of the 2C TCR occurs in the presence of H-2 L^d molecules and is characterized by deletion of DP thymocytes (23). As shown in Fig. 3, CD45RA and CD45RB_{high} expression are upregulated on 1B2^+ thymocytes in 2C H-2 D^d/b mice. However, CD45RA^{+} and CD45RB_{high} cells in H-2 D^d/b mice have a lower 1B2 intensity than CD45RA^{+} and CD45RB_{high} cells in positively selecting H-2 D^d/b mice (Fig. 3). Triple staining with anti-CD45, 1B2, and either CD4 or CD8 revealed that these cells were DN (99%) and DP (1%). Deletion of 1B2^+ cells is not dependent on CD8 expression since negative selection of this TCR still occurs in CD8^-/- H-2 D^d/b 2C transgenic mice (20a). CD45RA expression on 1B2^+ DN cells in H-2 D^b/b...
Figure 4. CD45RA expression on 2C transgenic thymocytes in CD8+/+ and CD8−/− mice. Thymocytes were stained with anti-CD45RA antibodies and 1B2 as described in Fig. 3. Shown is CD45RA vs. 1B2 expression on thymocytes from CD8+/+ and CD8−/− mice in H-2b/b and H-2k/b haplotypes. Percentages of CD45RA+ thymocytes were determined as described in Materials and Methods. Quadrant markers were set as described in Fig. 3. The mice in this analysis were 11–15 wk of age.

Discussion
CD45RB expression was examined on thymocytes from normal mice. While all thymocytes were CD45RB−, there was a small population (1–3%) with a higher intensity of CD45RB expression (CD45RBhi). CD45RBhi thymocytes, while present in all four thymic subsets defined by CD4 and CD8 expression, were predominant in the DN and CD8 SP subsets. Skewing towards the DN and CD8 SP subsets of expression of high molecular weight isoforms of CD45 has also been reported for CD45RA, CD45RC, and CD45RB (26–28). Double staining with CD45RB and TCR-α/β-specific antibodies revealed a population of CD45RBhi TCR-α/βhi thymocytes. CD45RA antibodies have also been shown to stain a small population of TCR/CD3hi thymocytes (27). However, in contrast to our findings, CD45RBhi staining was found on a population of thymocytes with an intermediate intensity of TCR-α/β expression (28).

Expression of CD45RA and CD45RB isomers was also examined on thymocytes from H-Y and 2C TCR transgenic mice in selecting (H-2b/b), nonselecting (H-2d/d for H-Y and H-2k/b for 2C), and deleting (H-2b/b male mice for H-Y and H-2d/d for 2C) backgrounds. It has been proposed that thymocytes expressing high molecular weight isoforms of CD45 represent a thymic generative lineage that maintains CD45RA expression throughout intrathymic differentiation (29). The observation that CD45RA+ and CD45RBhi TCR+ thymocytes were present among the DN, DP, and CD8 SP subsets in both transgenic models in positive selecting backgrounds may support this continuous lineage model whereby CD45RA and CD45RBhi are expressed during thymic development. However, in nonselecting backgrounds of the two different transgenic mouse models, DN and DP CD45RA+ and CD45RBhi thymocytes expressing high levels of the transgenic TCR were absent from both TCR transgenic mice, a finding that is in conflict with this model.

Our observations are consistent with an alternative model in which expression of CD45RA and CD45RBhi is induced by thymic maturation events. In both H-Y and 2C TCR transgenic models studied here, expression of CD45RA and CD45RBhi on transgene-positive thymocytes occurred only when the ligands for positive or negative selection were present. In the H-2b/b-selecting haplotype, CD45RA and CD45RBhi expression on T3.70hi thymocytes from female H-Y mice was observed on a subset of TCRhi CD8 SP and DP thymocytes, both of which are positively selected populations (9, 20). Similarly, in H-2b/b 2C transgenic mice, 1B2hi thymocytes expressing CD45RA and CD45RBhi belonged to the positively selected CD8 SP compartment and the DP compartment (23). 1B2hi DP thymocytes likely represent positively selected cells since they are not found in the nonselecting (H-2k/b) background of 2C transgenic mice (W. P. Fung-Leung, unpublished observations). In male H-2b/b H-Y transgenic animals, CD45RA and CD45RBhi expression was observed on CD8low T3.70hi cells, a subset shown to be dependent upon H-Y/H-2 D for development (22). T3.70hi DP thymocytes expressing CD45RA and CD45RBhi in male H-Y transgenics may represent thymocytes that have been positively selected on H-2 D or negatively selected by H-Y/H-2 D. Therefore, the finding that CD45RA and CD45RBhi expression was occurring on thymocytes that have been selected further supports the hypothesis that selection events result in CD45RA and CD45RBhi expression. It is interesting to note that in H-Y male mice homozygous for the CD8 mutation, CD45RBhi expression was observed on T3.70hi thymocytes. Although in the absence of CD8 expression, thymocytes expressing this TCR are not clonally deleted (20a), they may still upregulate CD45RBhi expression in the presence of H-Y/H-2 D.

Not all mature cells in a normal thymus are positive for CD45RA and CD45RBhi expression. Similarly, not all mature transgenic TCR+ CD8 SP thymocytes in either transgenic model are positive for CD45RA and CD45RBhi expression, although they express identical TCRs. One possibility is that CD45RA and CD45RBhi expression could be a transient event during development, perhaps occurring upon maturation from DP to mature SP thymocyte populations. The increase in the number of CD45RA+ or CD45RBhi cells among transgenic thymocytes as com-
pared with nontransgenic thymocytes may be due to the fact that in transgenic mice more thymocytes have been positively selected at any given time. This is supported by the observation that there are more CD8 SP and DP TCR high thymocytes in transgenic as compared with nontransgenic mice (9, 23, 30).

CD45RA and CD45RBJ high expression was also found on TCR high DN thymocytes in H-2 b/d H-Y and 2C transgenic mice. The presence of transgenic TCR high DN thymocytes has been reported in both transgenic models and their development is not dependent upon positive selection (9, 25). The observation that an interaction of CD8 with H-2 D b is required for positive selection of the H-Y TCR + cells (31) and that CD8 expression is required for positive selection of both H-Y and 2C TCR-bearing thymocytes (20a) renders it unlikely that CD45RA + - and CD45RBJ high -expressing TCR high DN thymocytes detected in H-2 b/d transgenic mice are the products of positive selection. An alternative hypothesis is that these cells may be thymic progenitor cells that express the transgenic TCR at an early stage, since in both rat and mouse thymic precursor activity can be detected within a population of DN thymocytes expressing high molecular weight CD45 (32, 33). This is also supported by data that in both transgenic models the transgene is expressed very early in development on DN thymocytes (25, 34).

In 2C H-2 b/d-deleting transgenic mice, 1B2 high thymocytes expressing CD45RA and CD45RBJ high were found almost exclusively within the DN subset. The observation that there were more 1B2 + DN thymocytes expressing CD45RA and CD45RBJ high (45 and 5 times more, respectively) in H-2 b/d mice as compared with H-2 b/d mice leads us to speculate that these TCR + DN thymocytes have had their TCRs engaged by the deleting ligand, H-2 L d. Since CD45RA and CD45RBJ high expression occurred on thymocytes with a lower intensity of TCR expression in H-2 b/d mice as compared with CD45RA + and CD45RBJ high 1B2 high thymocytes from H-2 b/d mice suggests that these cells have escaped negative selection by downregulating TCR expression. The possibility that these are cells that have been positively selected is ruled out by the fact that positive selection of this TCR requires CD8 expression (20a) and by the observation that these cells are present in H-2 b/d mice (V. A. Wallace, unpublished observations), where the ligand for positive selection is absent.

The differences in CD45RA and CD45RBJ high expression on thymocytes described in this report may be due to differential binding of the exon-specific mAbs as a result of conformational or glycosylation changes in the CD45 molecules during thymic development rather than differences in exon usage. However, the epitopes detected by the CD45 exon-specific mAbs used in these experiments are not dependent upon lymphocyte-specific glycosylation since they have been shown to stain CD45-transfected fibroblast lines and are not disrupted by the presence of other variable exons (16, 35; and V. A. Wallace, unpublished observations). While these results do not completely rule out that the antibodies recognize a glycosylated epitope, or that glycosylation on other exons may affect epitope recognition, the differential staining of thymocytes that we observe with these antibodies clearly correlates with selection events.

In conclusion, the data from normal thymocytes and from the transgenic mouse models suggest that thymic selection events may induce expression of CD45 isoforms containing variable exons. However, activation of peripheral T cells expressing high molecular weight CD45 isoforms results in exon switching to low molecular weight isoforms (11). This difference may arise because the molecular events associated with peripheral T cell activation may be different from those initiated during thymic selection. Alternatively, the goal of thymic selection is to produce naive T cells expressing high molecular weight CD45 (36). The functional implications of selection-induced upregulation of CD45 high molecular weight isoforms on thymocyte signaling and development need to be determined.

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Address correspondence to Tak W. Mak, Ontario Cancer Institute, Department of Medical Biophysics and Immunology, University of Toronto, 500 Sherbourne Street, Toronto, Ontario, M4X 1K9, Canada.

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