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Selective Regulation of TCR Signaling Pathways by the CD45 Protein Tyrosine Phosphatase during Thymocyte Development

Rustom Falahati* and David Leitenberg2*†

In CD45-deficient animals, there is a severe defect in thymocyte-positive selection, resulting in an absence of mature T cells and the accumulation of thymocytes at the DP stage of development. However, the signaling defect(s) responsible for the block in development of mature single-positive T cells is not well characterized. Previous studies have found that early signal transduction events in CD45-deficient cell lines and thymocytes are markedly diminished following stimulation with anti-CD3. Nevertheless, there are also situations in which T cell activation and TCR signaling events can be induced in the absence of CD45. For example, CD45-independent TCR signaling can be recovered upon simultaneous Ab cross-linking of CD3 and CD4 compared with cross-linking of CD3 alone. These data suggest that CD45 may differentially regulate TCR signaling events depending on the nature of the signal and/or on the differentiation state of the cell. In the current study, we have assessed the role of CD45 in regulating primary thymocyte activation following physiologic stimulation with peptide. Unlike CD3-mediated stimulation, peptide stimulation of CD45-deficient thymocytes induces diminished, but readily detectable TCR-mediated signaling events, such as phosphorylation of CD3ζ, ZAP70, linker for activation of T cells, and Akt, and increased intracellular calcium concentration. In contrast, phosphorylation of ERK, which is essential for positive selection, is more severely affected in the absence of CD45. These data suggest that CD45 has a selective role in regulating different aspects of T cell activation. The Journal of Immunology, 2008, 181: 6082–6091.

The CD45 protein tyrosine phosphatase plays a critical role in promoting T cell activation and development (1). Experiments in a variety of CD45-deficient T cell tumor lines, as well as data from CD45-deficient mice, have demonstrated that early events in TCR signal transduction pathways are severely impaired in the absence of CD45 (2–6). These observations are primarily attributed to the positive regulatory role of CD45 in promoting the activity of the src family kinase, Lck, which then helps to initiate TCR-mediated signal transduction pathways by inducing tyrosine phosphorylation of CD3ζ/ITAM residues and association and activation of the ZAP70 tyrosine kinase. CD45 promotes Lck activity by dephosphorylating the negative regulatory C-terminal tyrosine on Lck, maintaining Lck in an open active configuration (7–10). The importance of CD45-dependent regulation of Lck activity for T cell activation is further supported by experiments in which reconstitution of CD45-deficient cell lines and mice with constitutively active Lck is able to rescue the T cell activation and development deficit seen in the absence of CD45 (11–13).

Despite the recognized role of CD45 in promoting Lck activity and potentiating TCR signaling, CD45 also has been suggested to down-regulate T cell activation. In vitro experiments have indicated that CD45 has the potential to dephosphorylate TCR-associated CD3ζ and ZAP70, and to dephosphorylate the positive regulatory tyrosine phosphorylation site within the catalytic domain of Lck (14–16). It remains unclear how these apparently contradictory roles are regulated in vivo. One possibility is that CD45 plays a predominantly positive regulatory role before T cell activation by maintaining an active pool of Lck, but may also down-regulate ongoing TCR-dependent signaling cascades due to redistribution of CD45 in different signaling complexes or membrane compartments after T cell activation (17–20). Thus, the precise role of CD45 in regulating the duration and strength of different TCR-dependent signal transduction pathways remains uncertain. In particular, there has been very little reported on the role of CD45 in regulating T cell activation and TCR signal transduction in primary cells in response to stimulation by Ag and APCs.

There are several lines of evidence indicating that the dependence of CD45 for TCR signaling is not absolute, suggesting that CD45 may play a role in differentially regulating or tuning distinct parameters of T cell activation. As noted above, experiments in CD45-deficient tumor cell lines have shown defects in the response to CD3 Ab-mediated cross-linking in the absence of CD45 that is recovered by CD45 genetic reconstitution. However, the defects in stimulation are variable in different cell lines, suggesting that some CD45-independent TCR-dependent signaling events can also occur. For example, in the CD45-deficient BW thymoma cell line, high doses of immobilized anti-CD3 induce similar levels of IL-2 production when compared with CD45-reconstituted cell lines (21).

In vivo analysis of T cell development in CD45-deficient mice also suggests that CD45-independent T cell activation can occur. CD45-deficient mice have a near complete block in thymic development at the double-positive (DP) stage, indicating a failure in positive selection (22–25). However, the transition from double-negative to DP cells is less severely affected, suggesting that some

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3Abbreviations used in this paper: DP, double positive; LAT, linker for activation of T cells; pERK, phosphorylated ERK; PLC, phospholipase C; pMCC, moth cytochrome c peptide.
signaling through the pre-TCR is able to occur in the absence of CD45. This phenotype contrasts with the phenotype of mice deficient in other molecules involved in TCR signaling, such as Lck/Fyn, linker for activation of T cells (LAT), or SLP-76, where there is a defect in pre-TCR signaling resulting in a block in development at the double-negative stage (26–30). In addition, previous studies of CD45-deficient mice crossed with various TCR transgenic strains have evaluated T cell activation and development by assessing positive and negative selection in the thymus. In all of these studies, there has been a consistent block in positive selection regardless of the TCR transgenic strain (22–25). However, negative selection in male HY transgenic animals and also upon superantigen stimulation is reduced in efficiency, but readily detectable in CD45-deficient animals, indicating that TCR signals sufficient to mediate negative selection can occur in the absence of CD45 (22, 24). One possible interpretation of this data is that signaling in response to strong TCR signals that normally induce negative selection can occur independently of CD45, whereas lower avidity signals required for positive selection are more CD45 dependent. Alternatively, specific signaling pathways critical for positive selection may be more dependent on CD45 than signaling events that mediate negative selection.

Further evidence of CD45-independent TCR signaling comes from data that, in contrast to CD3 cross-linking, simultaneous Ab cross-linking of CD3 and CD4 induces biochemical signaling events in CD45-deficient cells similar to that seen in wild-type control cells (5, 31). Because physiologic stimulation with peptide typically involves coreceptor signaling, we hypothesized that CD45-independent signaling pathways may be more evident using this mode of stimulation.

In the current study, we have assessed the role of CD45 in regulating TCR signaling and thymic development in the AND TCR transgenic mouse, bred onto a homozygous I-Ek background. Previous studies in this system have indicated that the AND TCR interaction with self-peptides/I-Ek complex is relatively strong, resulting in a significant amount of negative selection compared with selection on other MHC backgrounds (32, 33). In addition, selection of mature, class II-restricted, CD8+ T cells can occur (although to a lesser extent than wild-type mice) in CD4-deficient mice, indicating that positive selecting signals are strong enough to occur in the absence of coreceptor signaling (33, 34). Despite the increase in strength of signal inherent in this system, we find that CD45 is absolutely required for positive selection of mature AND T cells. In these mice, the absence of CD45 yields a block in thymic development in the early DP stage similar to previous studies of CD45-deficient mice (22–24). Notwithstanding the block in development, biochemical analysis of TCR signaling pathways following agonist peptide stimulation indicates that TCR-dependent signal transduction events, as indicated by TCRζ, ZAP70, and LAT tyrosine phosphorylation, as well as calcium mobilization, are reduced in magnitude, but can be induced in the absence of CD45. However, we find that activation of the ERK MAPK pathway, which is essential for positive selection, is much more severely impaired in CD45-deficient thymocytes in response to peptide stimulation. These data suggest that CD45 may differentially affect TCR-mediated signal transduction events and provide a possible explanation for the block in thymocyte-positive selection seen in CD45-deficient animals.

**Materials and Methods**

**Mice**

CD45 exon 9 null mice were purchased from The Jackson Laboratory, and have been backcrossed 7–9 times onto the B10. Br background, and intercrossed with AND TCR transgenic mice (23, 32). Mice were bred and maintained at the George Washington University animal facility. Animal procedures conform to institutional animal protocol guidelines.

**Pep tide**

Moth cyt chro m e ε peptide (pMCC), VFAGLLKNNADLLAY LKQAKTT (aa 81–103), was synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory and purified by HPLC before use.

**Antibodies**

Polyc lonal rabbit anti-mouse TCRζ and ZAP70-specific Abs have been described previously (35). Anti-mouse CD4 (GK1.5), CD8α (53-6.72), CD69, CD3e, and Vαl were purchased from BD Biosciences in unlabeled or fluorescent conjugated forms. Anti-mouse LAT was purchased from Santa Cruz Biotechnology. Phosphospecific Abs to Lck, LAT, phospholipase C (PLC)-γ, Akt, ZAP-70, and ERK were purchased from Cell Signaling Technology. Pan-specific anti-phosphotyrosine Ab (4G10) was purchased from Upstate Biotechnology. Goat anti-rat Ig and HRP-conjugated goat anti-rabbit Ig were purchased from Jackson ImmunoResearch Laboratories.

**Preparation of DP thymocytes**

DP thymocytes were purified from total thymocytes from CD45+/-- and CD45-/- AND TCR transgenic mice by flow cytometry cell sorting with an Ab against CD8α (53-6.72). Purity of the recovered CD4+ CD8--TCRζαβ- DP thymocytes was >90%, as determined by flow cytometric analysis. To insure that the sorting process and bound CD8α Ab were not impacting the basal status or activation of the thymocytes, we compared unlabelled prestored and sorted cells. This comparison demonstrated similar induction of tyrosine phosphorylation between prestored and sorted cells, indicating that neither the sorting process nor the CD8α Ab had any observable impact on basal or postactivation phosphorylation status (data not shown).

**Cell stimulation**

For Ab-mediated stimulation, thymocytes were preincubated with 5 μg/ml anti-CD3 (2C11) and/or 1 μg/ml anti-CD4 (GK1.5) for 10 min on ice. Bound Abs were cross-linked with goat anti-rat Ig at 50 μg/ml and immediately incubated at 37°C for the indicated amount of time. For stimulation with peptides, T cell-depleted splenocytes were loaded with 50 μg/ml pMCC for 4 h at 37°C in HBSS supplemented with 5% FCS. The APCs were rapidly pelleted with thymocytes at 1:1 ratio and incubated at 37°C for the indicated amount of time, and then detergent-soluble lysates were prepared (34).

**Cell lysis, immunoprecipitation, and immunoblotting**

Cell lysates containing equivalent number of cells were prepared by lysis in TNE buffer (20 mM Tris (pH 7.5), 150 mM NaCl, and 5 mM EDTA,) supplemented with complete Mini protease inhibitors (Roche Applied Science), 1 mM Na3VO4, and either 1% Nonidet P-40 (Nonidet P-40) or 1% N-dodecyl-β-D-maltoside (Maltoside), as indicated. Western blot analysis was done following SDS polyacrylamide electrophoresis and transfer onto nitrocellulose paper (Bio-Rad). General and specific tyrosine phosphorylation was detected with indicated Abs, followed by goat anti-rabbit-coupled HRP (Jackson ImmunoResearch Laboratories). All of the immunoblots were visualized with the ECL chemiluminescent detection system, and images were taken on film. Where indicated, bands from immunoblots were quantified by densitometry (Molecular Dynamics), and the relative degree of phosphorylation was corrected for total amounts of each specific protein.

**Flow cytometric analysis of ERK activation**

Following peptide stimulation for the indicated times, the thymocytes were immediately labeled with fluorescent conjugated Abs to CD4 and CD8 on ice for 1 min. Intracellular ERK phosphorylation was evaluated using fluorescent conjugated anti-phospho-p44/42 MAPK (E10) (Cell Signaling Technology) following fixation and permeabilization using the Fix & Perm M fluorescent conjugated anti-phospho-p44/42 MAPK (E10) (Cell Signaling Technology).
results were expressed as changes in normalized fluorescence over time. The percentage of responding cells was determined by dividing the number of cells in the field whose fluorescence increased 3-fold or was sustained 3-fold for more than 3 min by the number of cells in the field. Ionomycin was added at a concentration of 666 ng/ml at the end of scanning as a positive control.

**CD69 expression assay**

Thymocytes from CD45\(^{+/+}\) or CD45\(^{-/-}\) AND TCR transgenic mice were stimulated in the presence of T cell-depleted splenocytes with indicated concentration of peptide and/or PMA and/or ionomycin. After an overnight incubation, expression of CD4, CD8, V\(\alpha1\), and CD69 was simultaneously evaluated via flow cytometry. All CD69 expression data presented are gated on CD4\(^+\)CD8\(^-\)V\(\alpha1\)\(^+\) population.

**Results**

**Differential induction of tyrosine phosphorylation following stimulation with anti-CD3 or anti-CD3/CD4 in CD45-deficient thymocytes**

It is well established that there is a profound defect in TCR signal transduction events following stimulation of CD45-deficient cell lines and thymocytes with Abs to CD3 (1). However, previous data using CD45-deficient tumor cell lines have suggested that the defect in signaling can be overcome by simultaneous cross-linking of both CD3 and CD4 (5, 31). This is presumably due to juxtaposition of CD3 and CD4, and to the ability of anti-CD4 Abs to cross-link CD4-associated Lck molecules, promoting autoposphorylation of the positive regulatory tyrosine within the catalytic domain of Lck, facilitating Lck activation. To confirm these findings in primary cells, we isolated thymocytes from CD45\(^{+/+}\) and CD45\(^{-/-}\) mice and assessed protein tyrosine phosphorylation following anti-CD3 cross-linking alone or anti-CD3 and anti-CD4 cross-linking in combination (Fig. 1). Similar to the previous studies, anti-CD3 cross-linking induces little, if any, tyrosine phosphorylation in CD45\(^{-/-}\) thymocytes when compared with CD45\(^{+/+}\) thymocytes (6). In contrast, there is no apparent defect in overall tyrosine phosphorylation or ERK activation in CD45-deficient cells upon simultaneous cross-linking with anti-CD3 and anti-CD4. Indeed, tyrosine phosphorylation of some proteins following anti-CD3 and anti-CD4 cross-linking or CD4 cross-linking alone is augmented in CD45-deficient thymocytes when compared with controls (see arrowheads at p36 and p120; Fig. 1).

**Thymic development in CD45-deficient AND TCR transgenic mice**

Because Ab engagement of the CD4 coreceptor promoted TCR-dependent signaling in CD45-deficient cells, we hypothesized that APC-mediated stimulation with agonist peptide/MHC class II complexes that interact with the TCR and coreceptor may similarly induce CD45-independent signaling. To evaluate Ag-specific signaling, we bred the CD45-deficient mice with the AND TCR transgenic mice that have specificity for a peptide from moth cytochrome c. As shown in Fig. 2, the CD45-deficient AND transgenic mice have a near complete block in thymic development at the DP stage similar to previous reports of other TCR transgenic strains (22–25). In wild-type (or CD45\(^{+/+}\)) AND TCR transgenic mice, there is a very strong selection bias evident for the development of single-positive CD4 T cells.

Previous reports describing thymocyte development in (non-TCR transgenic) CD45-deficient mice have described a decrease in total thymocyte number (22–24). This is in contrast to our results described in Fig. 2, in which AND TCR transgenic CD45-deficient mice typically have a modest increase in total thymocytes compared with wild-type or CD45 heterozygous littermates (Fig. 2B). This is a result of the high degree of negative selection that occurs in AND TCR transgenic mice on a
homozogous I-E\(^b\) background, resulting in a relatively small thymus (32, 33). These findings emphasize the fact that there is a very strong requirement for CD45 for positive selection and the development of single-positive mature T cells even when the positively selecting signal is relatively strong. In contrast, CD45 appears less important for pre-TCR signaling and development of DP cells.

**CD45-independent and -dependent signal following stimulation with peptide-pulsed APCs**

To assess CD45-dependent regulation of TCR signal transduction pathways following peptide stimulation, total thymocytes from CD45\(^{+/+}\) and CD45\(^{-/-}\) AND TCR transgenic mice were isolated and stimulated with pMCC-pulsed T-depleted spleen cells as APCs (Fig. 3A). Although induction of total tyrosine phosphorylation was less robust, the overall pattern of tyrosine phosphorylation was similar in cell lysates from CD45\(^{-/-}\) total thymocytes compared with CD45\(^{+/+}\) thymocytes. This is in contrast to the near undetectable tyrosine phosphorylation response to stimulation with Abs to CD3 (Fig. 1).

Although initiation of early tyrosine phosphorylation in response to agonist peptide signaling is evident in the absence of CD45, we went on to evaluate more downstream TCR-dependent signal transduction events. Specifically, activation of the ras/ERK signaling cascade is known to be critical for positive selection, and any defects in this pathway would correlate with the block in T cell development seen in the absence of CD45 (36). Indeed, when ERK activation was assessed following stimulation with agonist peptide (data are from the same blot shown in Fig. 3A), there was a marked defect in ERK activation in the absence of CD45 compared with CD45\(^{+/+}\) control thymocytes (Fig. 3B). These data suggest that ERK activation is relatively more dependent on CD45 than induction of tyrosine phosphorylation in other signaling molecules.

Because the difference in ERK activation between CD45\(^{+/+}\) and CD45\(^{-/-}\) thymocytes may be magnified due to the large number of mature TCR\(^{high}\), single-positive T cells from thymocytes of control animals, we also evaluated the induction of phosphorylated ERK (pERK) by intracellular staining and flow cytometric analysis, gating specifically on the DP cells. In agreement with the data in Fig. 3B, ERK activation is severely compromised in the CD45-deficient thymocytes following stimulation with agonist peptide-pulsed APCs (Fig. 3C). Low levels of ERK phosphorylation within a subset of the CD45-deficient cells are detectable within 2 min after activation; however, this is not sustained, and no evidence for ERK activation is seen at later time points. In comparison, CD45\(^{+/+}\) DP cells exhibit substantially higher and more sustained ERK activation when compared with CD45\(^{-/-}\) DP cells similar to the Western blot data (Figs. 3B and 4).

Note that the defect in ERK activation in CD45-deficient cells contrasts with the data in Fig. 1, in which ERK activation was induced in CD45-deficient thymocytes following CD3/CD4 Ab cross-linking. Thus, physiologic stimulation with agonist peptide appears to overcome defects in membrane-proximal TCR signal transduction events evident upon anti-CD3 stimulation, but does not fully restore T cell activation as seen upon anti-CD3 and anti-CD4 cross-linking.

**Selective regulation of ERK phosphorylation by CD45 in CD4\(^+/+\) CD8\(^{+/+}\) DP thymocytes following stimulation with peptide**

Further analysis of individual components of the TCR signal transduction cascade also suggests that there is a specific defect in ERK activation in the absence of CD45, whereas other TCR-dependent signaling events are relatively CD45 independent following peptide stimulation. To examine CD45-dependent regulation of specific biochemical signaling events following peptide stimulation, we performed a series of experiments using purified CD4\(^+/+\) CD8\(^+/+\) DP thymocytes from both CD45-deficient and control mice to alleviate the confounding single-positive CD4 T cell population in the control CD45\(^{+/+}\) mice.

Because Lck is the best defined substrate for CD45, we initially assessed CD45-dependent changes in Lck tyrosine phosphorylation using phosphospecific Abs to the negative regulatory C-terminal tyrosine (Lck-Y505), as well as the regulatory tyrosines within the kinase domain associated with kinase activity (Lck-Y394). Consistent with previous reports, Lck is hyperphosphorylated at both sites in the absence of CD45, although phosphorylation at the negative regulatory site is more severely affected (Fig. 4A) (16, 37).

To evaluate the functional activity of Lck in DP thymocytes, we stimulated the cells with agonist peptide and assessed tyrosine phosphorylation of TCR-associated \(\zeta\), ZAP-70, and LAT using a pan phosphotyrosine Ab to assess \(\zeta\)-chain phosphorylation and...
phosphospecific Abs for ZAP70 Y493 and LAT Y191. Induction of TCR/H9256, ZAP70, and LAT phosphorylation is dependent on Lck activity and would be predicted to be severely impaired in CD45-deficient animals (38). As seen in Fig. 4B, phosphorylation of TCR/H9256 p23, ZAP70, and LAT was all induced following peptide stimulation in the absence of CD45. Although CD45-deficient cells exhibited modest decreases in sustained Ag-induced tyrosine phosphorylation compared with control thymocytes, activation was readily detectable. In marked contrast, ERK phosphorylation was below detectable levels within 3 min after activation in the same experiment (Fig. 4B). Densitometric analysis reveals that the role of CD45 in promoting TCR-ζ p23, ZAP-70, and LAT phosphorylation is most evident at later time points, whereas phosphorylation early after stimulation (2 min in this experiment) is only reduced by 20–30% compared with control CD45+ cells (Fig. 4C). This is in contrast to the marked defect ERK activation at early time points and an even more pronounced defect (less than 1% of control) at later time points.

Importantly, in contrast to agonist peptide stimulation, basal levels of thymocyte TCR-ζ (p23), ZAP70, and LAT phosphorylation are dependent on Lck activity and would be predicted to be severely impaired in CD45-deficient animals (38). As seen in Fig. 4B, phosphorylation of TCR-ζ p23, ZAP-70, and LAT was all induced following peptide stimulation in the absence of CD45. Although CD45-deficient cells exhibited modest decreases in sustained Ag-induced tyrosine phosphorylation compared with control thymocytes, activation was readily detectable.

In marked contrast, ERK phosphorylation was below detectable levels within 3 min after activation in the same experiment (Fig. 4B). Densitometric analysis reveals that the role of CD45 in promoting TCR-ζ p23, ZAP-70, and LAT phosphorylation is most evident at later time points, whereas phosphorylation early after stimulation (2 min in this experiment) is only reduced by 20–30% compared with control CD45+ cells (Fig. 4C). This is in contrast to the marked defect ERK activation at early time points and an even more pronounced defect (less than 1% of control) at later time points.

Importantly, in contrast to agonist peptide stimulation, basal levels of thymocyte TCR-ζ (p23) phosphorylation were markedly reduced in the absence of CD45 (Fig. 4B, lane 1 vs lane 6). These data indicate that the low-avidity signaling that occurs in vivo in response to TCR interaction with self-peptides is dependent on CD45 expression. However, the current data indicate that agonist peptide signaling sufficient to induce saturated levels of CD3ε chain phosphorylation (p23) and ZAP70 and LAT phosphorylation can occur in the absence of CD45. The CD45-independent regulation of LAT phosphorylation compared with the CD45-dependent regulation of ERK phosphorylation is further shown upon stimulation with different doses of agonist peptide (Fig. 4D). In this experiment, purified DP thymocytes from CD45-deficient mice were stimulated with peptide-pulsed APCs (50 µg/ml, pMCC), as described in Fig. 3, for the indicated time periods and analyzed for specific phosphorylation of TCR-ζ, ZAP70, LAT, and ERK by Western blot using the indicated phosphospecific Abs. TCR-ζ phosphorylation was detected using a pan-specific phosphotyrosine Ab. The blots were then stripped and probed for total protein where indicated. C. Densitometric analysis of the data in B is graphically represented following normalization for T cells using the total Western blot data. Data shown are the percentage of the response of CD45-deficient cells compared with control CD45+ cells. D, Purified DP thymocytes from wild-type or CD45-deficient AND TCR transgenic mice were stimulated with peptide-pulsed APCs with the indicated dose of specific peptide for 5 min, and phosphorylation of LAT and ERK1/2 was detected using the indicated phosphospecific Abs. E. As in B, purified DP thymocytes from wild-type or CD45-deficient AND TCR transgenic mice were stimulated with peptide-pulsed APCs for the indicated periods of time, and activation of PLC-γ1, AKT, JNK1/2, and ERK1/2 was detected using the indicated phosphospecific Abs. The data shown in each panel are representative of at least two independent experiments for each molecule. Data within each panel are derived from the same set of cell lysates.
mice demonstrated a profound defect in ERK activation 5 min after stimulation with high doses of the agonist peptide, pMCC, whereas LAT phosphorylation was readily detectable. In contrast, wild-type control DP thymocytes were induced to activate ERK following stimulation with low doses of the agonist peptide similarly to high doses of peptide.

In additional independent experiments, we assessed other more downstream signal transduction events in addition to ERK in CD45-deficient thymocytes. Similarly to the data described above, PLC-γ, AKT, and JNK1/2 phosphorylation were all readily detectable in the absence of CD45, whereas ERK phosphorylation was markedly diminished in the same experiment (Fig. 4D). These data suggest a specific role for CD45 in the regulation of ERK activation, and are consistent with a model in which ERK activity is regulated independently of other downstream signaling events, such as AKT phosphorylation.

There does not appear to be a general defect in ERK activation in CD45-deficient thymocytes, because PMA stimulation of CD45-deficient cells or stimulation with anti-CD3/CD4 induces ERK activation similar to control mice (Fig. 1, data not shown). In total, these data suggest that ERK is capable of being activated in CD45-deficient cells, and indicate that CD45 serves as a critical positive regulator of TCR-dependent ERK activation following physiologic peptide stimulation.

**Diminished sustained calcium mobilization in individual CD45^- DP thymocytes following peptide stimulation**

In addition to ERK activation, TCR-induced increases in cytoplasmic calcium concentration are also required for efficient positive selection (39). To evaluate the role of CD45 in regulating calcium mobilization in individual DP thymocytes, cells were monitored for changes in calcium concentration by video laser microscopy following stimulation with peptide-pulsed APCs, as previously described (40). As shown in Fig. 5, both CD45^+ and CD45-deficient thymocytes were induced to increase intracellular calcium concentration in a peptide-dependent manner. However, upon analysis of the CD45-deficient thymocytes, there were ~50% fewer responding cells, and those cells were also less likely to exhibit a sustain increase in intracellular calcium.

**Defect in CD69 induction in CD45-deficient cells**

As suggested by the calcium mobilization data, CD45 may also affect other signaling events that are important for appropriate TCR-mediated induction of gene transcription and T cell development in addition to ERK activation. Because PMA was able to induce ERK activation in the CD45-deficient cells, we assessed whether PMA could complement the defect(s) in TCR signaling seen upon peptide stimulation and promote more downstream indicators of thymocyte activation. In these experiments, thymocytes were stimulated with agonist peptide alone or in combination with PMA, and induction of CD69 expression was assessed (Fig. 6). A relatively low dose of PMA that was sufficient to induce ERK activation, but insufficient to induce CD69 expression alone, was used in these experiments (Fig. 6). This dose of PMA was able to complement the induction of CD69 expression in combination with exogenous ionomycin, whereas higher doses of PMA induced CD69 expression in CD45-deficient cells in the absence of other signals (data not shown).

Previous reports have indicated that ERK activation is critical for stimulating CD69 expression, and that induction of CD69...
expression is associated with TCR signals that promote positive selection (41, 42). Thus, these experiments were based on the hypothesis that peptide stimulation alone would result in insufficient signaling to induce CD69 expression, whereas the addition of PMA (to activate ERK) to peptide-stimulated cells would correct the defect in ERK activation and stimulate an increase in CD69 expression. Contrary to this hypothesis, we observed that across a broad range of peptide concentrations, neither peptide alone, nor peptide with PMA, was able to induce CD69 expression in CD45−/−/− thymocytes with varied concentrations of peptide (serial 10-fold dilutions with highest concentration at 5 μg/ml) and/or PMA at 0.1 ng/ml. All CD69 expression data presented are gated on CD4+CD8+ Vα11+ population. These data are representative of three independent experiments.

Discussion

In this study, we have evaluated the role of CD45 in regulating TCR signal transduction events in primary thymocytes following physiologic stimulation with peptide ligand. Although reduced, membrane-proximal tyrosine phosphorylation events can be induced in DP thymocytes in response to agonist peptide stimulation in the absence of CD45. This contrasts with the response to anti-CD3 stimulation in which induction of tyrosine phosphorylation is more dependent on CD45 activity. There are several distinctions between stimulation with peptide and anti-CD3 that may explain the differential dependence on CD45. For example, TCR recognition of MHC class II/peptide complexes also involves ligation and cross-linking of CD4. Because Ab cross-linking of CD3 and CD4 together also stimulates CD45-independent T cell activation, it is likely that MHC class II interaction with CD4 may similarly promote CD4-dependent signaling events (most likely via Lck activation) in the absence of CD45. In addition to involving coreceptors, APC-dependent activation of T cells also involves a variety of other costimulatory and adhesive interactions that may also enhance or stabilize TCR signaling beyond that seen with anti-CD3 cross-linking.

The finding that TCRζ chain, LAT, and ZAP70 phosphorylation can be induced in CD45-deficient thymocytes following peptide stimulation suggests that Lck activity is not completely dependent on CD45. Many studies have shown that Lck activity is critical for TCRζ chain phosphorylation and ZAP70/TCR association and phosphorylation (38). CD45 is known to promote Lck activity by dephosphorylating the C-terminal tyrosine and maintaining Lck in an open and active conformation (9, 43). This promotes Lck kinase activity and also facilitates the adaptor role of Lck by freeing the Src homology 2 domain. Thus, the predominantly held model is that CD45 activity is critical for generating Lck-dependent membrane-proximal TCR signal transduction events. Previous studies in CD45-deficient cell lines using anti-CD3 stimulation have generally shown defects in early TCR signal transduction events and have supported this model (4, 6, 44, 45). However, our current data suggest that Lck activity is not entirely dependent on CD45. This is consistent
with previous studies in CD45-deficient cells that have suggested a complex role for CD45 in regulating Lck kinase activity both positively and negatively (16, 46). This is attributed to the finding that CD45 also dephosphorylates the positive regulatory tyrosine within the catalytic domain of Lck, resulting in little net change in Lck kinase activity (47). However, regardless of the role of CD45 in promoting Lck kinase activity, it is clear that CD45-dependent dephosphorylation of the C-terminal tyrosine is critical for promoting the adaptor function of the Lck Src homology 2 domain that may facilitate TCR signal transduction independently of Lck enzymatic activity (10, 48, 49). Our data suggest that the requirement for CD45 in promoting both the enzymatic and adaptor functions of Lck is not absolute.

An explanation for these results may be that distinct pools of Lck exist within the T cell and are differentially regulated by CD45. In support of this model, we have recently found that CD45 predominantly regulates tyrosine phosphorylation of co-receptor-associated Lck, whereas noncoreceptor-associated Lck phosphorylation is regulated independently of CD45 (50). These data support a model in which CD45 is required to maintain activity of co-receptor-associated Lck that in turn is critical for transmitting signals that activate ERK. In contrast, noncoreceptor-associated Lck may be able to function in the absence of CD45 and (at least in response to a strong agonist peptide) promotes phosphorylation of CD3 ITAMs, ZAP70, and LAT. In addition to different pools of Lck, there may also be different pools of CD45 that differentially regulate T cell activation. This model is consistent with recent data from Miceli and colleagues (51), indicating that distinct pools of CD45 have distinct roles in TCR signal transduction pathways. Similar to our findings, CD45 targeted to nonlipid raft domains was particularly critical for ERK activation, whereas CD45 expression was not required to induce LAT phosphorylation. In contrast, CD45 constitutively targeted to lipid-raft domains had an overall negative effect on both ERK and LAT phosphorylation.

One possible interpretation of our data is that there is a quantitative defect in signal strength in the absence of CD45. In this view, CD45-deficient thymocytes may receive partial signals sufficient to induce early tyrosine phosphorylation events, but which are insufficiently strong or sustained to promote more downstream signal transduction sufficient for ERK activation. In contrast to this interpretation are other data indicating that maximal ERK activation can be induced with low doses of agonist peptide or upon low-avidity interactions with the TCR using partial agonist peptides (52). Indeed, the stringent requirement of CD45 for ERK activation in the presence of LAT and ZAP70 phosphorylation, is the opposite of the signaling phenotype seen following TCR stimulation with low- potency partial-agonist peptides. Low-avidity partial-agonist peptides typically induce sustained ERK activation while failing to induce significant ZAP70 or LAT phosphorylation (53–55).

Overall, these data support the concept that ERK activation may be regulated independently of other parameters of TCR signaling, and that CD45 has a qualitative role in regulating distinct signal transduction events. The precise molecular basis for this differential regulation ERK activation by CD45 remains unclear. However, it is interesting to speculate that it may be related to previous reports examining the role of Lck SH3 domain in regulating T cell activation and development. The signaling phenotype in CD45-deficient thymocytes is remarkably similar to cells that express a mutated Lck SH3 domain (W97ALck), in which there is a similar selective defect in ERK activation following TCR stimulation, whereas ZAP70 and LAT phosphorylation are relatively unaffected (56, 57). In addition, thymocytes from knock-in mice with a mutated Lck SH3 domain have a relatively mild defect early in thymocyte development and progress through the pre-TCR checkpoint to DP cells, but have a more severe defect in positive selection similar (though less pronounced) to CD45-deficient mice (57). In Lck SH3 domain mutant cell lines, the defect in ERK activation has been attributed to a defect in Raf activation in the Golgi, whereas initial Ras activation is not severely affected (58). Our current data suggest that CD45 may also be particularly important for this role of Lck. Furthermore, our data suggest that coreceptor-associated Lck that is regulated by CD45 may have a specialized role in facilitating ERK activation.

Because ERK activation is required for the development of single-positive T cells, the defect in ERK activation correlates well with the block in positive selection seen in CD45-deficient mice. In addition, the ability to activate LAT independently of CD45 may also explain why pre-TCR signaling and transition from double-negative to DP cells is less affected in CD45-deficient thymocytes, whereas pre-TCR signaling is more severely compromised in mice deficient in Lck/Fyn or LAT (26–30). However, although there was a severe defect in ERK phosphorylation in the absence of CD45, pharmacologic activation of ERK with low doses of PMA in combination with peptide stimulation did not rescue the defect in CD69 expression present in the CD45-deficient thymocytes. This may be due to the nonphysiologic nature of PMA signaling, but also may indicate that there are defects in signal transduction events in addition to ERK activation in the absence of CD45. This is consistent with earlier data indicating that constitutive activation of the ras/MAPK pathway is not sufficient to promote mature T cell development of CD4+CD8+ thymocytes (59).

In summary, our results indicate that the generation of different TCR signal transduction pathways in developing thymocytes is not equally dependent on CD45 activity. This is particularly evident upon stimulation with a strong agonist peptide, in which early TCR signal transduction events can be induced in the absence of CD45 expression. These results are similar to recent reports evaluating immunoreceptor signal transduction in CD45-deficient B cells and NK cells (60–62). Upon BCR cross-linking or following ligation of activating NK cell receptors, early tyrosine phosphorylation events are induced in the absence of CD45, sufficient to promote selective aspects of both B and NK cell activation. In the presence of T cell help, CD45-deficient B cells undergo initial Ag-dependent activation in vivo and germinal center formation, but this response is abrogated with the block in positive selection due to defects in B cell survival. Similarly, CD45-deficient NK cells can be induced to exhibit cytotoxic function, but have severe defects in cytokine and chemokine production. Also, similarly to CD45-deficient thymocytes, both NK cells and B cells have severe defects in ERK activation. In total, these data identify CD45 as a critical, but selective regulator of signal transduction events that impacts on immune cell development and effector function.

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References


