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CD45 Down-Regulates Lck-Mediated CD44 Signaling and Modulates Actin Rearrangement in T Cells


The tyrosine phosphatase CD45 dephosphorylates the negative regulatory tyrosine of the Src family kinase Lck and plays a positive role in TCR signaling. In this study we demonstrate a negative regulatory role for CD45 in CD44 signaling leading to actin rearrangement and cell spreading in activated thymocytes and T cells. In BW5147 T cells, CD44 ligation led to CD45 and Lck clustering, which generated a reduced tyrosine phosphorylation signal in CD45− T cells and a more sustained, robust tyrosine phosphorylation signal in CD45+ T cells. This signal resulted in F-actin ring formation and round spreading in the CD45+ cells and polarized, elongated cell spreading in CD45− cells. The enhanced signal in the CD45− cells was consistent with enhanced Lck Y394 phosphorylation compared with the CD45+ cells where CD45 was recruited to the CD44 clusters. This enhanced Src family kinase-dependent activity in the CD45− cells led to PI3K and phospholipase C activation, both of which were required for elongated cell spreading. We conclude that CD45 induces the dephosphorylation of Lck at Y394, thereby preventing sustained Lck activation and propose that the amplitude of the Src family kinase-dependent signal regulates the outcome of CD44-mediated signaling to the actin cytoskeleton and T cell spreading. The Journal of Immunology, 2008, 181: 7033–7043.
example, cross-linking of L-selectin or CD44 activates Lck (32, 33) and both LFA-1 and CD44 cross-linking can induce protein kinase C-regulated migration of T cells (34, 35). Overall, CD44 exhibits overlapping functions with the selectins and integrins that together contribute to leukocyte extravasation. Signals transmitted to the cell as a consequence of their interactions may facilitate leukocyte adhesion and subsequent migration.

CD44 cross-linking activates Lck (33) and can lead to an increase in intracellular calcium levels (36) and activation of protein kinase C (35). CD44 can associate with Lck and Fyn in the low-density sucrose fraction after solubilization in 1% Brij 58 or Triton X-100, although the amount varies with the cell line and solubilization conditions (21, 37). Localization to this low-density fraction is equated with localization in cholesterol and sphingolipid-rich membrane domains referred to as lipid rafts. The low level of CD45 in this fraction (~1%) and the hyperphosphorylation of Lck in this fraction (38) led to the suggestion that CD45 is excluded from lipid rafts. However, other data do not support this suggestion and instead suggests that CD45 is dynamically associated with these detergent resistant domains (39, 40). This suggests that localization of CD45 may be one mechanism to control its activity. Presently, little is known about how the tyrosine phosphatase activity of CD45 is regulated. In this study, we investigate how CD45 regulates CD44-mediated T cell signaling events leading to cell adhesion and cell spreading. Previous work indicated that CD44-mediated signaling leading to Pyk2 phosphorylation and elongated T cell spreading was mediated by Src family kinases and negatively regulated by CD45 (21). In this study, we compare CD44-mediated signaling events leading to T cell adhesion in the presence and absence of CD45 and propose a role for CD45 in down-regulating Lck activity and in determining the duration and strength of signals that affect actin rearrangement and T cell spreading. CD44 clustering in CD45− T cells leads to a small tyrosine phosphorylation signal, the formation of an F-actin ring and round spreading, whereas in the absence of CD45, a robust tyrosine phosphorylation signal led to more extensive, elongated cell spreading. This Src family kinase-dependent tyrosine phosphorylation in the CD45− T cells led to activation of phospholipase C (PLC)γ and PI3K, which were both required for elongated cell spreading.

Materials and Methods

Abs and reagents

Rat anti-mouse CD44 mAbs, IM7, KM81, and KM201 from American Type Culture Collection (ATCC) were used as well as the following rabbit anti-antiserum: J1WBB against the cytoplasmic domain of mouse CD44 (21), R54-3B against residues 34–150 of Lck (41), R02-2 against the cytoplasmic domain of mouse CD45 (42), and pY394 specific for phosphorylated Y394 of Lck (43). Soluble ICAM-1 was a gift from Dr. F. Takei (Terry Fox Laboratory, Vancouver, British Columbia, Canada).

Materials

BW5147 T cells were harvested from C57BL/6 mice, and CD45−/− mice were passaged through a strainer to generate a single cell suspension. (1–2 × 10^6) were treated with RBC lysis buffer (0.83% w/v NH₄Cl, 10 mM Tris-HCl (pH 7)) before being labeled with biotinylated anti-CD19 (15 μg), CD11b (7.5 μg), NK1.1 (15 μg), and Ter119 (7.5 μg) Abs and anti-biotin MicroBeads were added before passing through LS columns (Miltenyi Biotec) according to the manufacturer’s instructions, to enrich for double negative (DN) thymocytes. Thymocytes were then cultured in stimulation medium (RPMI 1640, 10% heat-inactivated FBS, 0.055 mM 2-ME, 10 mM HEPEs) at 2 × 10^6/ml containing 12.5 ng/ml PMA (Sigma-Aldrich) and 250 ng/ml ionomycin (Sigma-Aldrich) in the presence of 20 U/ml recombinant mouse IL-2 (R&D Systems). On the fourth day, the medium containing PMA and ionomycin was removed and replaced with fresh medium containing IL-2 only. Activated DN thymocytes were used for cell spreading assays on the seventh day.

Lymph nodes (LN) harvested from C57BL/6 and CD45−/− mice were pooled and performed according to the University Animal Care Committee and Canadian Council of Animal Care Guidelines.

Cell spreading assay and cell length measurement

The anti-CD44 mAb, KM81, was immobilized on 96-well plates (tissue culture-treated plates, Falcon or Nunc) at 40 μg/ml in PBS overnight at 4°C, essentially as described (21). Cells (5 × 10^4) in 50 μl of spreading medium (DMEM with 0.1% heat-inactivated FBS) were added for various times at 37°C before fixation with a final concentration of 4% paraformaldehyde for 20 min at room temperature. Light images of cells were taken with a Nikon Coolpix 950 mounted on a Nikon inverted microscope (Eclipse TS100) with a 20× or 40× objective and imported into Adobe Photoshop. Measurement of cell length for BW5147 T cells was calibrated with a microscope stage ruler. Cell length for activated primary thymocytes and T cells was measured from F-actin-labeled confocal images imported into NIH ImageJ. Polarized cells were identified as cells showing a significant asymmetrical, elongated phenotype and were distinguished from nonpolarized cells, which were predominantly round. Three independent experiments were performed (two independent experiments for LN T cells), and at least 85 cells were measured. Significance was determined with the unpaired Student’s t test.

Labeling for confocal microscopy

A total of 5 × 10^4 T cells or 1.5 × 10^5 activated DN thymocytes were incubated on Ab or BSA-coated chamber slides (Lab-Tek) in 150 μl of spreading medium (DMEM or RPMI 1640 with 0.1% heat-inactivated FBS) for 30 min or 2 h at 37°C then fixed as mentioned. Alternatively, BW5147 T cells were pretreated with 20 μM PP2, 20 μM LY294002, 0.5 μM U73343, or 0.5 μM U73122 for 30 min at 37°C before being incubated and fixed on the coated slides. The cells were washed and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature, then incubated with 1% BSA at room temperature for 30 min to prevent non-specific binding. BW5147 T cells were incubated for 1 h with primary Abs in 1% BSA/PBS or overnight in 15 μl of 10 U/ml Alexa Fluor 488-conjugated phalloidin in PBS. Cells were washed three times, incubated with corresponding Alexa Fluor-conjugated Abs for 1 h. Activated primary cells were incubated for 1.5 h with 100 μl of 10 U/ml Alexa Fluor 488-conjugated phalloidin in PBS. Cells were washed three times with PBS.
before being mounted in 90% v/v glycerol/2.5% v/v DABCO (1,4-diazabicyclo-[2, 2, 2]octane) in PBS (Sigma-Aldrich). For double labeling, the staining was conducted in a sequential manner with four Ab-labeling steps. Control labeling without the primary Ab was included to ensure no cross-reactivity occurred between the secondary Abs.

**Image collection with confocal microscopy**

Images of labeled BW5147 T cells were captured with Bio-Rad Radiance 2000 or Plus on a Nikon Eclipse 2000i or Zeiss Axiovert, respectively, using a 63x oil-immersion objective. The images were collected with Kalman collection filter (2X) with a step size of 0.3 μm. Image size was 512 x 512 pixels covering the dimension of 162 x 162 μm. Images of labeled BW5147 T cells, activated T cells or thymocytes were captured with Olympus Fluoview FV1000 using a 60X or 100X objective, with a step size of 0.19 μm covering dimensions of 212 x 212 μm or 127 x 127 μm. For double labeling experiments, images were collected with the same settings in a sequential manner. For any given experiment, the same laser power and gain controls settings were used to ensure consistent signal intensity. Cells from random fields were collected and analyzed from at least three independent experiments.

**Image processing**

Fluorescent images were processed in either NIH Image or NIH ImageJ. One of the images close to the interface between the cells and the slide was selected from the stack of confocal images and reopened in Adobe Photoshop. Alternatively, a projection of five images close to the interface was made to enhance signal-to-noise ratio. The image/adjustment/level command was used to adjust image contrast.

**Immunoprecipitation and Western blotting**

Cells were pretreated with or without 20 μM PP2 or LY294002 at 37°C for 30 min before being added to a 6-well plate (Nunc) containing immobilized CD44 Ab, and incubated at 37°C for various times. Then, 5 x 10^5 cells/ml were lysed with 250 μl of ice-cold 2X lysis buffer (5% Triton X-100, 50 mM Tris-HCl (pH 7.2), 700 mM KCl, 10 mM EDTA (pH 8.0), 2.5 mM sodium orthovanadate, 1 mM sodium molybdate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM PMSF). Lysates were centrifuged and 1 μl of anti-PLCγ1 Ab, or 2.5 μl of R54-3B was added at 4°C for 1 h. Then 20 μl protein A beads (Repligen) were added for 1 h. The beads were washed with lysis buffer and samples resolved in a 7.5% polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Immobilon P; Millipore). For anti-phosphotyrosine detection, blots were incubated with 1:5000 4G10; phosphoY505 of Lck was detected by incubation with 1:1000 anti-phospho Lck (Y505) Ab or 0.5% BSA in TBS-T (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% v/v Tween 20) for 1.5 h at room temperature. After several brief washes with TBS-T, the blot was incubated with 1:5000 anti-phospho IgG-HRP or protein A-HRP for 1 h. The blot was then washed several times with TBS-T and bands visualized with ECL or ECLplus (Amersham Biosciences). Membranes were stripped in 50 mM glycine (pH 2.5), 150 mM NaCl, 0.1% v/v Nonidet P-40 for 30 min at room temperature, then washed with TBS-T and reprobed with 1:200 anti-PLCγ1 or 1:5000 anti-Lck in 5% skim milk/TBS-T and 1/5000 anti-rabbit IgG-HRP or protein A-HRP. For whole cell lysate blots, polyvinylidene fluoride membranes of cell lysates from 2.5 x 10^6 cells were probed with 1/10000 phospho-Akt (S473) and 1/5000 anti-rabbit IgG-HRP. Alternatively, membranes were probed with 1/200 anti-Csk Ab and 1/5000 protein A-HRP before stripped and reprobed with 1/5000 anti-β-actin Ab and 1/5000 anti-mouse IgG-HRP.

**Results**

**CD45 affects the outcome of CD44-mediated actin rearrangement and cell spreading in BW5147 T cells**

Comparison of the extent of cell spreading between CD45^- and CD45^+ BW5147 T cells on immobilized CD44 mAb, KM81, revealed that the CD45^- cells underwent a small, but significant increase of 11% in cell diameter and the cells exhibited round spreading (Fig. 1A). No spreading was observed with cells on immobilized BSA. In the absence of CD45, the cells adopted an elongated phenotype resulting in a much larger increase in cell length of 69% (Fig. 1A). Cells started to spread at 5 min and the majority of cells had spread by 30 min. A similar pattern of spread was observed with immobilized LFA-1 mAb (TIB213) and immobilized recombinant soluble ICAM (data not shown).

Confocal microscopy of the spread cells labeled with Alexa Fluor 488-conjugated phalloidin revealed that the CD45^- T cells formed an F-actin ring close to the interface between the cell and the slide (Fig. 1B, top panel). A time course indicated that the rings started to form by 5 min and were present in the majority of cells (70%, n = 92) by 30 min. The actin rings were still present at 2 h (63%, n = 136). Often the F-actin ring would be located within the perimeter of the cell, which may represent the edge of the contact zone between the cell and the slide. In CD45^- T cells, the F-actin ring was not observed. Instead, actin filaments were observed at the sides of the elongated cell (Fig. 1B, top panel). The F-actin cables were always aligned with the longitudinal axis of the cell, suggesting a directional axis. None of these F-actin structures were observed when the T cells were incubated on immobilized BSA. This suggests that the presence of CD45 can influence F-actin formation and cell shape induced by CD44-mediated signaling.

**Src family kinases mediate actin rearrangement in both CD45^- and CD45^+ T cells**

We had previously shown that Src family kinase activity is required for elongated spreading in CD45^- T cells (21). To determine whether Src family kinase activity was also required for F-actin rearrangement and cell spreading, BW5147 T cells were pretreated for 30 min with 20 μM of the Src family kinase inhibitor, PP2, then immobilized on CD44 mAb for 2 h and the formation of F-actin examined after staining with Alexa Fluor 488-conjugated phalloidin. Treatment of the CD45^- cells with PP2 resulted in the loss of F-actin rings, with the F-actin staining resembling that of cells on immobilized BSA (Fig. 1B, bottom panels). CD45^- cells also lost the characteristic F-actin staining, which became more punctate as the cells lost their elongated shape and rounded up. This indicates that Src family kinase activity is required for CD44-induced actin rearrangement in both the CD45^- and CD45^+ T cells. However, the difference in F-actin formation and cell shape between the CD45^- and CD45^- T cells suggests that CD45 can influence the outcome of the Src family kinase-mediated signal.

**CD45 affects the extent of CD44-mediated cell spreading and cell polarization in activated DN thymocytes and T cells**

To determine whether CD45 also affected CD44-mediated cell spreading in primary cells, we compared the spreading of CD44^+, CD4 and CD8 DN thymocytes, and LN T cells isolated from CD4^-/- (46) and CD45^-/- mice. Because it had previously been demonstrated that CD44-mediated spreading only occurs with activated T cells (47), DN thymocytes and T cells were activated with PMA and ionomycin before spreading on the immobilized CD44 mAb (Fig. 2). Analysis of DN1 and DN2 populations ex vivo indicated that CD44 was expressed at slightly higher levels on the CD4^-/- cells (data not shown), suggesting that CD45 may also influence the expression of CD44. However, there was no significant difference in CD44 expression after stimulation and at day 7, the majority of activated DN thymocytes were CD44^- and levels were comparable between CD45^-/- and CD45^-/- cells (Fig. 2A). Both the CD45^-/- and CD45^-/- activated DN thymocytes spread on the CD44 mAb and induced CD44 clustering (data not shown). As with the CD45^-/- BW5147 T cells, the CD45^-/- activated DN thymocytes spread more extensively on the CD44 mAb than the CD45^-/- cells (Fig. 2B) and this was significant after quantitation (Fig. 2C, left). The CD45^-/- activated DN thymocytes not only spread significantly longer than the CD45^-/+
FIGURE 1. CD44-mediated cell spreading and actin rearrangement in CD45^+ and CD45^- T cells. A, CD45^+ and CD45^- BW5147 T cells were incubated on immobilized BSA or CD44 mAb for 2 h. Cells were then fixed with 4% paraformaldehyde and photographed. The longest length of the cells was measured. Graph shows the average length with the SD, and data represent the average length of over 250 cells taken from three separate experiments. **p < 0.01 for a significant difference between the length of the two different samples. B, CD45^+ and CD45^- BW5147 T cells were untreated or treated with 20 μM PP2 and incubated on CD44 mAb or BSA for 2 h and fixed with 4% paraformaldehyde. F-actin was labeled with Alexa Fluor 488-conjugated phalloidin after the cells were permeabilized with 0.1% Triton X-100. The confocal images are a single 0.3-μm slice close to the interface between the cells and the immobilized CD44 mAb. Images are representative of 150 cells examined over three experiments. Actin rings were observed in 61% of the CD45^+ cells and data not shown). Approximately 80% of CD45^+ T cells (n = 152) and 89% of CD45^- T cells (n = 175) had clusters of CD44 at 30 min, indicating that their formation was independent of the presence of CD45. Incubation of the cells on BSA significantly reduced the number of cells showing CD44 clusters and latrunculin A, which prevents actin polymerization, significantly more polarized than the CD45^- T cells (Fig. 2C). The CD45^-/- activated DN thymocytes spread round or round with a slight extension or polarization. To examine the effect of CD45 on F-actin formation in the spread thymocytes, cells were immobilized on CD44 mAb for 30 min and then labeled with Alexa Fluor 488-conjugated phalloidin. In keeping with the round spreading, F-actin was primarily localized at the periphery of the cell in the majority of CD45^-/- activated DN thymocytes (Fig. 2D). This contrasts with the CD45^-/^- cells where the majority of cells had localized F-actin accumulation at one or both ends of the cell. No significant F-actin organization was observed when the cells were plated on BSA (data not shown).

Although there are greatly reduced numbers of peripheral T cells in the CD45^-/- mice, we sought to determine whether this observation also occurred in activated T cells. LN were isolated from multiple CD45^+^+ and CD45^-/- mice and T cells were enriched for by negative selection (see Materials and Methods). All the T cells expressed CD44 and the levels were enhanced upon activation with PMA and ionomycin. Activated CD45^+^+ and CD45^-/- T cells expressed comparable levels of CD44, yet, as with the T cell line and DN thymocytes, showed distinct spreading and polarization ability in response to the ligation of CD44. Activated CD45^-/- T cells spread significantly longer and were significantly more polarized than the CD45^-/+- T cells (Fig. 2E). F-actin labeling in the activated T cells also revealed distinct differences between the CD45^-/+- and CD45^-/- cells (Fig. 2F). Similar to the CD45^-/- BW5147 T cells and activated CD45^-/- DN thymocytes, CD45^-/- T cells showed a cortical distribution of F-actin, forming a ring around the periphery of the cell. In contrast, F-actin was more heterogeneously distributed in the CD45^-/- cell and was often enriched in cell protrusions at one end of the cell. Small filamentous spikes of F-actin were also often observed protruding from one end of the cell. Together, these data establish a role for CD45 in regulating CD44-mediated cell spreading and F-actin rearrangement in activated primary CD44^-^+ DN thymocytes and peripheral T cells.

**CD44 and Lck are recruited into clusters in both CD45^+ and CD45^- T cells upon CD44 engagement**

To investigate how CD45 can affect the outcome of Src family kinase mediated F-actin rearrangement and cell spreading, we investigated the temporal and spatial organization of Src family kinases in CD45^-^+ and CD45^-/- T cells spread on immobilized CD44 mAb. Antiserum against the cytoplasmic domain of CD44 was used to label CD44. Confocal microscopy revealed that at 5 min, CD44 had redistributed into clusters of CD44 at 30 min, indicating that their formation was independent of the presence of CD45. Incubation of the cells on BSA did not induce cluster formation. Treatment of the T cells with latrunculin A, which prevents actin polymerization, significantly reduced the number of cells showing CD44 clusters and...
also reduced cluster size, suggesting that F-actin polymerization promoted cluster formation (data not shown).

Lck was also recruited into clusters in a similar time frame as CD44, suggesting an intimate association between CD44 and Lck. Over 80% of both CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 T cells (n = 100 in both cases) had recruited Lck into clusters by 5 min (Fig. 3B). Clustering is known to activate Lck and this response was consistent with Src family kinase-dependent actin rearrangement occurring in both the CD45<sup>+</sup> and CD45<sup>-</sup> cells. However, it also suggests that even though Lck is hyperphosphorylated at the negative regulatory tyrosine (Y505) in the CD45<sup>-</sup> T cells (4), it is still active upon clustering of CD44. Co-labeling of CD44 and Lck in both CD45<sup>+</sup> and CD45<sup>-</sup> T cells demonstrated their colocalization in the clusters (Fig. 3D).

Because the Src family kinase Fyn resides in the low-density sucrose fraction along with CD44 and Lck, and can coprecipitate with CD44 (21), its distribution was also examined. Interestingly, by 5 min only 25% of CD45<sup>+</sup> cells (n = 68) had recruited Fyn into clusters, whereas 74% of CD45<sup>-</sup> cells (n = 84) had recruited Fyn (Fig. 3C). By 30 min, 48% of the CD45<sup>-</sup> T cells (n = 93) and 91% of the CD45<sup>-</sup> T cells (n = 67) had clusters of Fyn. This result

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**FIGURE 2.** CD44-mediated cell spreading and F-actin rearrangement in activated primary CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 thymocytes and T cells. A, CD44 expression on activated CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 DN thymocytes determined by flow cytometry and shown as the average percentage of CD44-expressing cells and mean fluorescence intensity (MFI) ± SEM from six independent experiments. B, Phase-contrast image of activated DN thymocytes incubated on immobilized BSA or CD44 mAb for 30 min. C, Average length ± SD of activated CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 DN thymocytes for 30 min. At least 85 cells were counted over three independent experiments. ***, p < 0.001. Percentage of polarized cells ± SEM from four independent experiments.**, p < 0.05. D, F-actin labeled activated CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 DN thymocytes after immobilization on CD44 mAb for 30 min. The confocal images are a stack of five 0.19-μm horizontal slices close to the interface between the cells and the immobilized CD44 mAb. Images are representative of cells examined over three experiments. Scale bar represents 10 μm. E, Average cell length ± SD of at least 221 cells for CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 activated LN T cells immobilized on CD44 mAb (left). ***, p < 0.001. The percentage of polarized cells ± SEM obtained from six mice over two independent experiments is also shown (right). **, p < 0.01. F, F-actin labeled, activated CD45<sup>+/</sup>/H11001 and CD45<sup>-/</sup>/H11002 LN T cells after immobilization on CD44 mAb for 30 min. The confocal images are a stack of five 0.19-μm horizontal slices close to the interface between the cells. The image is one representative of two experiments.

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and 97% of CD45\(^+/\)H11002\(^{-}\) cells were fixed with 4% paraformaldehyde after incubation on immobilized BSA for 30 min or CD44 mAb for 5 and 30 min. Confocal images are one horizontal 0.3-µm slice close to the interface between the cells and the immobilized mAb, as described in Materials and Methods. A, CD44 was then labeled with J1WBB, which recognizes the cytoplasmic domain of CD44. B, Lck was labeled with 488-conjugated anti-rabbit IgG Ab. C, Shows the labeling of Fyn. D, Double labeling of cells with CD44 (green) and Lck (red) mAbs after 30 min of incubation on immobilized CD44 mAb. Confocal image of five 0.19-µm slices is shown. Scale bar represents 10 µm in each image. These are representative images observed in over 65 cells in each observation, from at least three independent experiments.

indicated that the presence of Lck in CD44 clusters was independent of CD45 expression, whereas Fyn recruitment was delayed and less efficient in the CD45\(^{-}\) T cells.

**Sustained induction of phosphotyrosine is only observed in the CD45\(^{-}\) T cells**

To determine the outcome of Lck and Fyn clustering with CD44 in the CD45\(^{+}\) and CD45\(^{-}\) cells, the cells were colabeled with an anti-CD44 cytoplasmic domain and anti-phosphotyrosine Ab (Fig. 4). At 5 min, clusters of phosphotyrosine were observed in CD45\(^{-}\) T cells, whereas virtually no staining was observed in the CD45\(^{+}\) cells. By 30 min, more phosphotyrosine was present in the CD45\(^{-}\) T cells and the majority of the CD44 clusters (97%, n = 175) were positive for phosphotyrosine. In contrast, only the occasional spot of phosphotyrosine was seen in the CD45\(^{+}\) T cells. This observation indicates that CD44 clustering leads to robust, sustained tyrosine phosphorylation in the CD45\(^{-}\) T cells. Induction of tyrosine phosphorylation in the CD45\(^{+}\) T cells occurred at a much lower intensity and to a much lesser extent than in the CD45\(^{-}\) T cells. This finding indicates that CD45 negatively regulates the CD44-induced tyrosine phosphorylation signal.

**CD45 is recruited to the CD44 clusters**

To investigate how CD45 might be down-regulating CD44 signaling, its localization was monitored upon CD44-mediated signaling. Surprisingly, like CD44 and Lck, CD45 was also recruited to the clusters within 5 min and was still associated at 30 min (Fig. 5A). In addition to localizing in the clusters, CD45 also formed a ring around the immobilized cell, similar to that observed for F-actin. Double labeling confirmed that CD45 colocalized to the same clusters as CD44 and Lck (Fig. 5, B and C). This colocalization of the tyrosine phosphatase CD45 with the CD44/Lck clusters is consistent with the reduced phosphotyrosine levels observed in the CD45\(^{-}\) T cells (Fig. 4).

**Y394 phosphorylation associated with sustained Lck activation does not occur in CD45\(^{+}\) T cells**

Localization of CD45 to CD44 clusters could decrease phosphotyrosine levels directly or indirectly by dephosphorylating and inactivating Lck. To investigate the latter, CD45\(^{+}\) and CD45\(^{-}\) T cells were labeled with the phosphospecific Lck Y394 antiserum, which recognizes Lck phosphorylated at the positive regulatory site. At 30 min, Y394 phosphorylation was clearly observed in the CD45\(^{-}\) T cells and was detectable in the clusters of ~38% (n = 276) of the cells (Fig. 6A). In contrast, very little phosphorylated Y394 was observed in the CD45\(^{+}\) T cells and no signal was observed in the clusters (0%, n = 147). Given the colocalization of CD45 and Lck with the CD44-induced clusters and the absence of phosphospecific Lck Y394 staining in these clusters, we conclude that CD45 is down-regulating CD44-induced signaling events by inducing the dephosphorylation of Lck Y394 and thereby limiting Lck activation.

To further determine that this robust Lck activation, evidenced by Y394 phosphorylation, was due to the absence of...
CD45 and not to changes in Y505 phosphorylation by another phosphatase, the phosphorylation state of Y505 was monitored upon CD44-mediated cell spreading. Fig. 6B shows that as expected, Lck was hyperphosphorylated at Y505 before CD44 signaling and that the level did not change significantly upon CD44-mediated cell spreading. This is in agreement with other work that shows that Lck can be phosphorylated at both Y394 and Y505 in the absence of CD45 (17) and that despite Y505 phosphorylation, Y394 phosphorylation is a necessary prerequisite and accurate predictor of Lck kinase activity (48, 49). Finally, we confirmed that the differences in Lck Y505 phosphorylation were consistent with the loss of CD45 and were not due to any differences in expression of Csk in the CD45 + and CD45 − T cells (Fig. 6C).

Src family kinase dependent activation of the PI3K pathway and PLC is required for the formation of actin filaments and elongated spreading in the CD45 − T cells

To further understand why Lck activation and sustained tyrosine phosphorylation led to enhanced, elongated cell spreading in the CD45 − T cells, we examined downstream signaling events. Upon TCR ligation, Src family kinase activation can result in the activation of several downstream signaling molecules including PI3K, PLCγ1, and MAPK (50–52). To determine whether these pathways were activated upon CD44 signaling, we first examined activation of the PI3K pathway by monitoring AKT phosphorylation. Phospho-AKT (S473) was induced upon CD44 ligation in the CD45 − T cells (Fig. 7A) to a much greater extent than observed in the CD45 + T cells. This induction was significantly reduced by 20 μM PP2 (Fig. 7A), indicating that activation of the PI3K pathway was Src family kinase dependent.

Although linker for activation of T cells (LAT) and MAPK (ERK1/2) phosphorylation are key events in TCR signaling, these proteins were not phosphorylated upon CD44 ligation in either the CD45+ or CD45− cells (data not shown). In TCR signaling, LAT phosphorylation facilitates the recruitment and activation of PLCγ1, which initiates the release of intracellular calcium (53). As CD44 signaling has been shown to involve calcium (36), it was of interest to determine whether PLCγ1 was phosphorylated in the absence of LAT phosphorylation. CD44 ligation induced the transient tyrosine phosphorylation of PLCγ1 in the CD45 +, but not the CD45− T cells (Fig. 7B). This phosphorylation was inhibited by 20 μM PP2 but not 20 μM LY294002 (Fig. 6C), indicating a dependence on Src family kinase activity, but not PI3K activity. Thus both PI3K and PLCγ1 were activated to a much greater extent in the CD45 − T cells, in a Src family kinase dependent manner.
To determine whether either of these two proteins accounted for the difference in F-actin organization and cell spreading observed between the CD45⁺/H11001 and CD45⁺/H11002 T cells, the cells were treated with PI3K or PLC inhibitors. Interestingly, treatment with the PI3K inhibitor (20 μM LY294002) resulted in the formation of F-actin rings and round spreading in the CD45⁺/H11002 T cells, similar to that observed in the CD45⁺/H11001 cells (Fig. 8). The inhibitor did not affect actin ring formation in the CD45⁺/H11001 T cells, although in both cases the actin rings were smaller in the presence of the PI3K inhibitor. Thus the PI3K inhibitor prevented elongated cell spreading and induced round spreading in the CD45⁺ T cells. Addition of U73122, an inhibitor of PLC, but not the inactive analog U73343, also prevented the elongated cell spreading and formation of F-actin cables in the CD45⁺ T cells and led to the formation of F-actin rings and round spreading (Fig. 8, bottom panels). The inhibitor had no observable effect on F-actin ring formation and round spreading in the CD45⁺ T cells. This experiment indicates that PLC and PI3K activities are both necessary, but alone not sufficient, for F-actin rearrangement and elongated cell spreading in the CD45⁺/H11002 T cells.

Overall, the recruitment of CD45 to CD44 clusters induces transient Lck activation that results in a signal that leads to the formation of an F-actin ring and round spreading. In the absence of CD45, unchecked Lck activation induces sustained protein tyrosine phosphorylation and activation of PI3K and PLCγ that results in directed F-actin formation and elongated T cell spreading.

FIGURE 7. Induction of phosphorylation of AKT and PLCγ1 upon immobilization on CD44 mAb. CD45⁺ and CD45⁻ T cells were treated with or without 20 μM PP2, incubated on immobilized CD44 mAb for the indicated times, and cell lysates were prepared and resolved by SDS-PAGE, as described in Materials and Methods. A, Whole cell lysates (the equivalent of 2.5 × 10⁶ cells) from CD45⁺ and CD45⁻ T cells were loaded in each lane and probed with phospho-S473-specific AKT Ab (pS473, top) before being stripped and reprobed with AKT Ab (bottom). Bands were detected with ECL or ECLplus. B and C, PLCγ1 was immunoprecipitated from 5 × 10⁶ CD45⁺ and CD45⁻ T cells, and samples were probed for phosphotyrosine with 4G10 (pTyr, top), stripped and reprobed with anti-PLCγ1 (bottom). L indicates lysate. In C, CD45⁻ T cells were immobilized on CD44 mAb for the time indicated after being treated or not with 20 μM PP2. Cells incubated on BSA for 30 min before PLCγ1 immunoprecipitation (B) or lysate only (L) are indicated. CD45⁻ T cells immobilized on CD44 mAb for the time indicated are shown with medium alone (M), DMSO (D), or 20 μM LY294002 (LY). A 10-min incubation on BSA (B) is also indicated.

FIGURE 8. CD44-mediated F-actin rearrangement in the presence of PI3K and PLC inhibitors. CD45⁺ and CD45⁻ T cells were pretreated with or without 20 μM LY294002, 0.5 μM U73122, or 0.5 μM U73343 for 30 min before incubation on immobilized CD44 mAb for 2 h. The cells were then fixed and stained with Alexa Fluor 488-conjugated phalloidin. Pictures shown are single 0.3-μm slices of confocal images close to the interacting surface with immobilized mAb. These data are representative of three independent experiments. Scale bar represents 10 μm.
signaling by dephosphorylation of Lck Y394. This response is consistent with the ability of CD45 to directly dephosphorylate Y394 of Lck in vitro (54) and with the hyperphosphorylation of Y394 in CD45− T cells (20).

CD44 signaling had a different outcome on F-actin formation and cell spreading, depending on the presence or absence of CD45 in BW5147 T cells and in activated primary thymocytes and T cells. Signaling in the presence of CD45, attributable to transient Lck activation, resulted in peripheral F-actin ring formation and predominantly round spreading, whereas in the absence of CD45, CD44 ligation led to directed or localized F-actin rearrangement and elongated, polarized cell spreading. In the CD45− BW5147 T cells this elongated cell spreading was attributed to sustained Lck activation leading to PI3K and PLCγ activation.

The transient and weak vs sustained and robust signaling from the same receptor leading to different outcomes is reminiscent of other key receptors in T cells. For example, a weak TCR signal in the thymus can lead to positive selection, whereas a stronger signal leads to negative selection (55, 56). Also, costimulatory signals can sustain transient TCR signals (57), the duration of which is important for T cell activation and IL-2 production (58–60). Interestingly, these signals also rely on Src family kinase activity and are also affected by the presence and absence of CD45.

In addition to the transient and sustained nature of CD44-induced signaling in the CD45+ and CD45− cells, there are other differences that may contribute to the different signaling outcomes. One relates to Lck phosphorylation at Y505 in the CD45− T cells (4). Although this does not prevent Lck from initiating a signal upon CD44 clustering, it may recruit Src homology 2 domain containing proteins that modify the subsequent signal. Secondly, perhaps due to the sustained nature of Lck activation, Fyn is recruited more efficiently to the clusters in the CD45− T cells and so its activity may also contribute to the different signaling outcomes observed between the CD45+ and CD45− cells. Indeed, in T cells, Fyn is known to phosphorylate Pyk2 (61), a focal adhesion-related kinase that has been implicated in cell spreading (reviewed in Ref. 62). Consistent with this observation, CD44 preferentially induces Pyk2 phosphorylation in the CD45− cells (21). Fyn may also link to the actin cytoskeleton via ADAP/SLAP/Fyb, a Fyn associated molecule, which is important in TCR-induced integrin-mediated clustering and adhesion (63). Thus Fyn activation may be important in transmitting the CD44 signal to the cytoskeleton. Fyn recruitment to the clusters lagged Lck recruitment, suggesting that Fyn may be a downstream effector of Lck. This sequential Src family kinase activation has recently been observed upon TCR activation, where TCR and CD44 cross-linking lead to rapid Lck activation, which is required for subsequent Fyn activation (64).

Immobilization of T cells on a planar surface containing TCR Abs has been used as a model system to mimic T cell activation occurring via TCR engagement and has been shown to generate an actin ring (65, 66). Formation of this actin ring in response to the TCR signal was dependent upon tyrosine kinase activation and the raft resident adaptor molecule, LAT (65). Although there are some similarities between CD44 and TCR signaling in that they both involve Lck and Fyn and can lead to actin ring formation, CD44 signaling did not induce LAT phosphorylation (data not shown). Although LAT was not phosphorylated upon CD44 ligation, PLCγ1 was activated in the CD45− T cells and this was important, together with PI3K for disruption of the F-actin ring and elongated cell spreading. Although the activation of both PLCγ1 and the PI3K pathway was Src family kinase dependent, the activation of the PI3K pathway was not CD44-specific as incubation of CD45− T cells on BSA also induced AKT phosphorylation (data not shown). Phosphorylation may be due to the basal level of Src family kinase activity present in these cells. Nevertheless, activation of the PI3K pathway alone was insufficient to induce actin rearrangement or cell spreading on BSA.

In order for cells to migrate, cell elongation and polarization has to occur. Fanning et al. (35) recently reported that activated human lymphocytes, which exhibit polarized cell spreading, do migrate on immobilized CD44 Ab. In neutrophils and dictyostelium, PI3K has been shown to play a major role in establishing cell polarity and directional migration (67, 68). Whether this finding is also true for lymphocytes remains to be established (69). Consistent with our data, PLCγ1 has recently been shown to be important for integrin-mediated cell spreading and elongation in endothelial cells and was also required for subsequent cell motility (70). In addition to cell polarization, a certain amount of cell adhesion is needed for migration, however adhesion has to be finely regulated as either too little or too much adhesion can prevent migration. Given that CD45 can negatively regulate CD44-mediated T cell adhesion and is redistributed upon directional cell migration in neutrophils (71), CD45 may also modulate T cell migration.

Actin ring formation has been reported to occur upon TCR stimulation (65, 66) and TCR induced actin rearrangement is crucial for immune synapse formation between a T cell and an APC upon Ag recognition (72, 73). It is possible that CD44 signaling at the immune synapse may augment this type of actin rearrangement. Although CD44 has been reported to have costimulatory activity (74), the localization of CD44 at the immune synapse and its contribution toward actin rearrangement at the immune synapse are not known. In this study we have shown that CD44 signaling resulting in F-actin rearrangement and cell spreading is mediated by Src family kinases and modulated by CD45. Unlike its accepted role in the dephosphorylation of Lck at Y505, which is required for its effective participation in TCR signaling events, we show a role for CD45 in the dephosphorylation of Lck at Y394, which prevents sustained Lck activation thereby modulating actin rearrangement and cell spreading in T cells.

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Disclosures

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