# CD43 interaction with ezrin-radixin-moesin (ERM) proteins regulates T-cell trafficking and CD43 phosphorylation

J. L. Cannon[a,*], P. D. Mody[a], K. M. Blaine[a], E. J. Chen[b], A. D. Nelson[a], L. J. Sayles[a], T. V. Moore[c], B. S. Clay[a], N. O. Dulin[a], R. A. Shilling[a,c], J. K. Burkhardt[b], and A. I. Sperling[a,c]

[a]Section of Pulmonary and Critical Care Medicine, Department of Medicine, University of Chicago, Chicago, IL 60637; [b]Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and University of Pennsylvania School of Medicine, Philadelphia, PA 19104; [c]Committee on Immunology, University of Chicago, Chicago, IL 60637

**ABSTRACT** **Cell polarization is a key feature of cell motility, driving cell migration to tissues. CD43 is an abundantly expressed molecule on the T-cell surface that shows distinct localization to the migrating T-cell uropod and the distal pole complex (DPC) opposite the immunological synapse via association with the ezrin-radixin-moesin (ERM) family of actin regulatory proteins. CD43 regulates multiple T-cell functions, including T-cell activation, proliferation, apoptosis, and migration. We recently demonstrated that CD43 regulates T-cell trafficking through a phosphorylation site at Ser-76 (S76) within its cytoplasmic tail. Using a phosphorylation-specific antibody, we now find that CD43 phosphorylation at S76 is enhanced by migration signals. We further show that CD43 phosphorylation and normal T-cell trafficking depend on CD43 association with ERM proteins. Interestingly, mutation of S76 to mimic phosphorylation enhances T-cell migration and CD43 movement to the DPC while blocking ERM association, showing that CD43 movement can occur in the absence of ERM binding. We also find that protein kinase Cθ can phosphorylate CD43. These results show that while CD43 binding to ERM proteins is crucial for S76 phosphorylation, CD43 movement and regulation of T-cell migration can occur through an ERM-independent, phosphorylation-dependent mechanism.**

**Monitoring Editor**
Josephine Clare Adams
University of Bristol

Received: Jul 13, 2010
Revised: Jan 7, 2011
Accepted: Jan 19, 2011

## INTRODUCTION

T-cells are crucial effectors of the immune response, providing help to B-cells for antibody production as well as mediating cellular immunity necessary for pathogen clearance. Before activation, naïve T-cells circulate in and out of lymph nodes, constantly surveying for antigen (von Andrian and Mempel, 2003). This surveillance is critical for T-cell function, and entry into lymph nodes is mediated by a stepwise molecular cascade leading from selectin rolling by CD62L to up-regulation of the integrin lymphocyte function–associated antigen 1 (LFA-1) by the chemokine receptor CCR7 (Mempel *et al.*, 2006). Several other molecules have also been shown to be important for T-cell migration (Sinclair *et al.*, 2008).

Our laboratory recently demonstrated a role for the T-cell surface molecule CD43 in regulating T-cell migration. CD43 is a heavily glycosylated mucin-like protein highly expressed on leukocytes (Carlsson *et al.*, 1986). Using a competitive in vivo assay, we found that CD43 positively regulates T-cell trafficking specifically to lymph nodes (Mody *et al.*, 2007). The cytoplasmic region of CD43 is rich in serines and threonines that are highly conserved among rat, mouse, and human (>70% amino acid homology), strongly suggesting that the CD43 intracellular domain (ICD) can support signal transduction. We found that the serine 76 (S76; S347 of the full-length molecule) of the CD43 cytoplasmic tail is phosphorylated, and this phosphorylation mediates normal T-cell trafficking (Piller *et al.*, 1989; Mody

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10-07-0586) on February 2, 2011.

*Present address: Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM 87131.

Address correspondence to: J. L. Cannon (jucannon@salud.unm.edu).

Abbreviations used: CFSE, carboxyfluorescein succinimidyl ester; CMAC, CellTracker™ Blue CMAC (7-amino-4-chloromethylcoumarin); DPC, distal pole complex; ERM, ezrin-radixin-moesin; FL, full-length; GFP, green fluorescent protein; GSK-3β, glycogen synthase kinase 3β; GST, glutathione S-transferase; ICAM-1, intercellular adhesion molecule 1; ICD, intracellular domain; IL, interleukin; IS, immunological synapse; LFA-1, lymphocyte function–associated antigen 1; OVA, ovalbumin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SDF-1α, stromal cell–derived factor 1α; siRNA, small interfering RNA; TCR, T-cell receptor; Tg, transgenic

*et al.*, 2007). While it is clear that S76 phosphorylation is crucial for CD43 regulation of T-cell trafficking, the signals and molecules regulating this phosphorylation remain unknown.

A key feature of CD43 function in T-cells is its specific localization within the migrating and activated T-cell. On activation, many molecules are localized to the T-cell antigen–presenting cell interface, termed the immunological synapse (IS) (Dustin, 2009). In contrast, CD43 localizes to the opposite pole of the IS, termed the distal pole complex (DPC) (Sperling *et al.*, 1998; Allenspach *et al.*, 2001; Delon *et al.*, 2001). DPC formation is important for T-cell function, including production of the cytokines interleukin-2 (IL-2) and interferon-γ. Other proteins also localize to the DPC, including phosphatases and inhibitors of T-cell activation, suggesting that the DPC may sequester proteins inhibitory to full T-cell activation (Cullinan *et al.*, 2002).

CD43 also redistributes to the uropod in migrating T-cells and neutrophils (del Pozo *et al.*, 1995). The specific localization of CD43 to the DPC and the uropod are dependent on CD43 interaction with the ezrin-radixin-moesin (ERM) proteins. ERM proteins play an important role in the plasma membrane–actin filament association and microvilli formation (Tsukita and Yonemura, 1997; Yonemura *et al.*, 1999). CD43 interacts with ERM proteins via a tripeptide KRR sequence within the CD43 cytoplasmic tail (Yonemura *et al.*, 1993, 1998). Perturbing CD43 interaction with ERM proteins either by mutation of the CD43–ERM binding site or by overexpression of ERM proteins results in the loss of CD43 localization and IL-2 production (Tong *et al.*, 2004).

In this study, we investigate the intracellular mechanisms that lead to CD43 phosphorylation and regulation of T-cell trafficking. We find that CD43 phosphorylation is regulated by chemokines and requires its association with ERM. In the absence of ERM association, CD43 phosphorylation is decreased, but a constitutively phosphorylated mutant of CD43 partially rescues defects in CD43 localization and T-cell trafficking without increasing direct binding to ERM proteins. These results show that CD43 localization and CD43 effects on T-cell migration are mediated by two distinct molecular sites within CD43, one at the ERM interaction site and the other at the site of phosphorylation.

## RESULTS

### CD43 S76 phosphorylation is modulated by signals that control migration

We have previously demonstrated that CD43 is phosphorylated at S76 in the cytoplasmic tail, and T-cells unable to phosphorylate S76 show a defect in lymph node accumulation, suggesting that signaling pathways that regulate S76 phosphorylation would, in turn, regulate T-cell migration (Mody *et al.*, 2007). To begin the study of the molecular mechanisms that control S76 phosphorylation, we generated an antibody that specifically detects only phosphorylated S76 within CD43 (Supplemental Figure 1). Using this antibody, we asked whether S76 phosphorylation can be modulated by specific stimuli known to regulate T-cell trafficking in vivo, including the chemokine CCL21, which binds CCR7 to induce T-cell entry into lymph nodes. We also investigated the role of the extracellular domain of CD43, which we previously showed to be important in T-cell migration (Mody *et al.*, 2007).

We treated purified T-cells with CCL21 or anti-CD43 cross-linking as well as the phorbol 12-myristate 13-acetate (PMA) ester as a positive control as we and others had previously found total CD43 phosphorylation increased with PMA (Mody *et al.*, 2007). In agreement with our previous finding, we see that S76 is phosphorylated in resting naïve T-cells and phosphorylation is dramatically increased two- to fourfold upon stimulation with PMA (Figure 1). CCL21 also

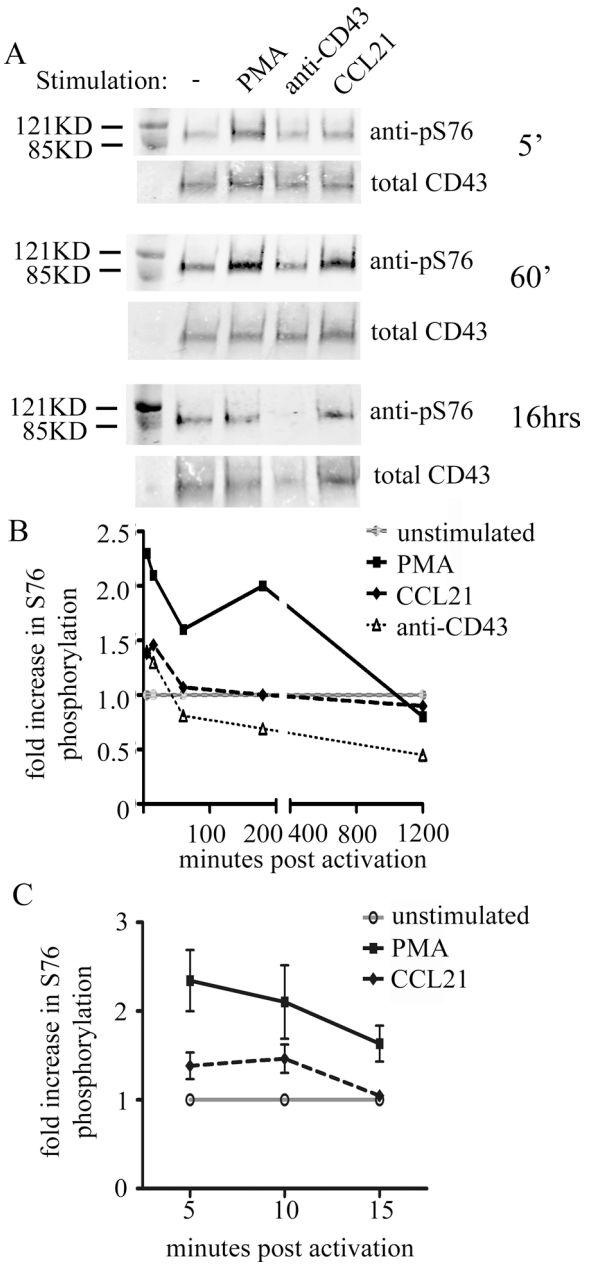


FIGURE 1: CD43 S76 phosphorylation is modulated by PMA, chemokine, and CD43 cross-linking. (A–C) T lymphoblasts were activated by PMA, CCL21, or α-CD43 (R260) for indicated times; lysed; analyzed on SDS–PAGE; and blotted for phospho-S76 and total CD43 on the same membrane. The molecular weight markers are visible only in one channel of the dual channel detection on the Li-Cor Odyssey. (B) Quantitation of S76 phosphorylation from (A) normalized to total CD43. (C) Quantitation of S76 phosphorylation in response to PMA and CCL21 from an average of three independent experiments.

consistently increases S76 phosphorylation, although not to the extent seen with PMA (Figure 1, B and C; 50% vs. 200–400%). Similar increases in S76 phosphorylation were seen using other chemokines such as stromal cell–derived factor 1α (SDF1α) (unpublished data). Increased S76 phosphorylation can be detected as early as 5 min, and decreases back to baseline by 60 min (Figure 1B). CD43 cross-linking induced decreased S76 phosphorylation at late time points, beginning at 60 min (Figure 1B) and continuing to decline to 16 h

(Figure 1, A and B). S76 phosphorylation does not change significantly upon T-cell receptor (TCR) signaling and integrin engagement (unpublished data). Together these data demonstrate that phosphorylation at S76 is modulated by migration signals including chemokines and CD43 ligand binding.

### CD43 association with ERM proteins regulates S76 phosphorylation

CD43 function in T-cells depends on its movement to the uropod of migrating cells and to the DPC upon T-cell activation through interaction of a tripeptide KRR sequence with the ERM family of cytoskeletal regulatory proteins (Yonemura *et al.*, 1998). When the KRR is mutated to NGG, CD43 cannot bind to ERM proteins and CD43 movement to the DPC and the uropod is blocked (Tong *et al.*, 2004). Because of the importance of the CD43–ERM association in CD43 function, we hypothesized that ERM binding would also regulate S76 phosphorylation. We assessed S76 phosphorylation using transgenic (Tg) mice that expressed either full-length CD43 (CD43-FL) or the NGG mutant of CD43 (CD43-NGG) to abolish ERM association. CD43-FL and CD43-NGG Tg mice express CD43 in all T-cells (see Supplemental Figure 2).

We purified T-cells from CD43-FL and CD43-NGG Tg mice and tested S76 phosphorylation. We found that PMA and CCL21 increased S76 phosphorylation in CD43-FL T-cells similar to wild-type T-cells (Figure 2A). In contrast, CD43-NGG T-cells show blunted S76 phosphorylation in response to PMA and CCL21 compared with CD43-FL T-cells (Figure 2, A and B). Because CD43 expression levels were slightly lower in CD43-NGG Tg T-cells compared with CD43-FL T-cells (Supplemental Figure 2), we quantitated S76 phosphorylation by normalizing to total CD43 levels. After normalization, we find that CD43-FL T-cells increased S76 phosphorylation almost twofold to CCL21 while CD43-NGG T-cells show no increase in S76 phosphorylation (Figure 2B, middle). CD43-NGG T-cells do respond to PMA, but the response is dramatically decreased compared with CD43-FL T-cells (Figure 2B, left). The difference in S76 phosphorylation between CD43-FL and CD43-NGG T-cells is not seen in response to CD43 cross-linking (Figure 2B, right). To ensure that the differences in S76 phosphorylation are not due to developmental differences in the CD43-FL and CD43-NGG Tg mice, we also tested S76 phosphorylation in CD43-deficient T-cells retrovirally transduced with CD43-FL or CD43-NGG. We find the same results as those seen in T-cells from CD43-FL and CD43-NGG Tg mice (unpublished data).

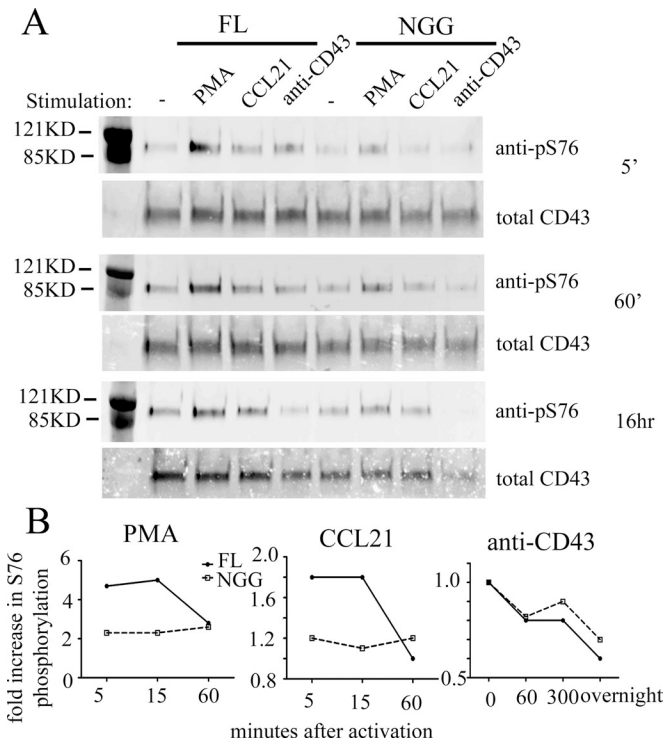


FIGURE 2: Failure to bind ERM proteins decreases CD43 S76 phosphorylation. (A and B) T-cells from Tg mice expressing CD43-FL or CD43-NGG were purified; activated with PMA, CCL21, or anti-CD43 (R260) for indicated times; lysed; and blotted for phospho-S76 and total CD43 (S11). (B) Quantitation of S76 phosphorylation shown in (A) normalized to total CD43.

The cytoplasmic tail of CD43 was recently shown to translocate to the nucleus, and previous studies have suggested that this translocation may depend on the same KRR site that regulates CD43–ERM binding (Andersson *et al.*, 2004; Seo and Ziltener, 2009). To ensure that the decrease in S76 phosphorylation seen in CD43-NGG mutants was due to ERM binding instead of other effects, we generated T-cells deficient in ERM proteins and tested for S76 phosphorylation. T-cells express ezrin and moesin, and deletion of ezrin and moesin represents deletion of the ERM family in T-cells; these cells were used previously to show that ezrin and moesin act together to mediate IL-2 production and proximal T-cell signaling (Shaffer *et al.*, 2009). We generated ezrin/moesin double-deficient T-cells by silencing moesin in ezrin-deficient T-cells ($Ez^{-/-}Mo^{SiM}$). We compared CD43 S76 phosphorylation in response to PMA and CCL21 in ezrin/moesin double-deficient T-cells with T-cells that express both ezrin and moesin ($Ez^{+/+}Mo^{SiC}$). $Ez^{-/-}Mo^{SiM}$ express ~20–25% of wild-type levels of moesin (Figure 3A, top). We find that while CD43 S76 phosphorylation in response to PMA in the ezrin/moesin double-deficient T-cells is similar to wild-type T-cells, CCL21-induced phosphorylation in ezrin/moesin double-deficient cells is blocked, similar to that seen in CD43-NGG mutants (compare Figures 2B and 3B). The lack of CCL21-induced S76 phosphorylation in both CD43-NGG and $Ez^{-/-}Mo^{SiM}$ T-cells demonstrates that ERM proteins play a crucial role in regulating CD43 S76 phosphorylation in response to CCL21.

### ERM proteins regulate CD43 effects on T-cell migration

We previously showed that CD43 S76 phosphorylation is important in regulating T-cell migration, and we now find that CD43 binding to ERM proteins regulates S76 phosphorylation. This led us to hypothesize that CD43 interaction with ERM proteins should also regulate T-cell trafficking to lymph nodes. We first utilized an in vitro transwell migration system to assay migration to specific stimuli involved in T-cell entry into lymph nodes, specifically CCL21-induced migration via CCR7. We asked whether CD43-NGG mutants migrate normally in response to CCL21. Briefly, CD43-deficient T-cells transduced with CD43-FL or the CD43-NGG mutant were differentially fluorescently labeled. Both populations were then combined and allowed to migrate toward the chemokine CCL21 together within the same well. The differentially labeled CD43-FL and CD43-NGG migrated cell populations were analyzed by flow cytometry and the ratio of CD43-FL to CD43-NGG populations calculated. As the input ratio of FL:NGG populations differed from experiment to experiment (see Supplemental Figure 5 for raw percentages), we normalized the migration of FL- or NGG-expressing T-cells to the input ratio in each experiment to assess the effect of CCL21 (Figure 4A). We find that CD43-NGG T-cells show defects in migrating to CCL21 compared with CD43-FL T-cells, as shown by the increased ratio of FL:NGG above 1, reflecting the fact that more CD43-FL cells migrated to

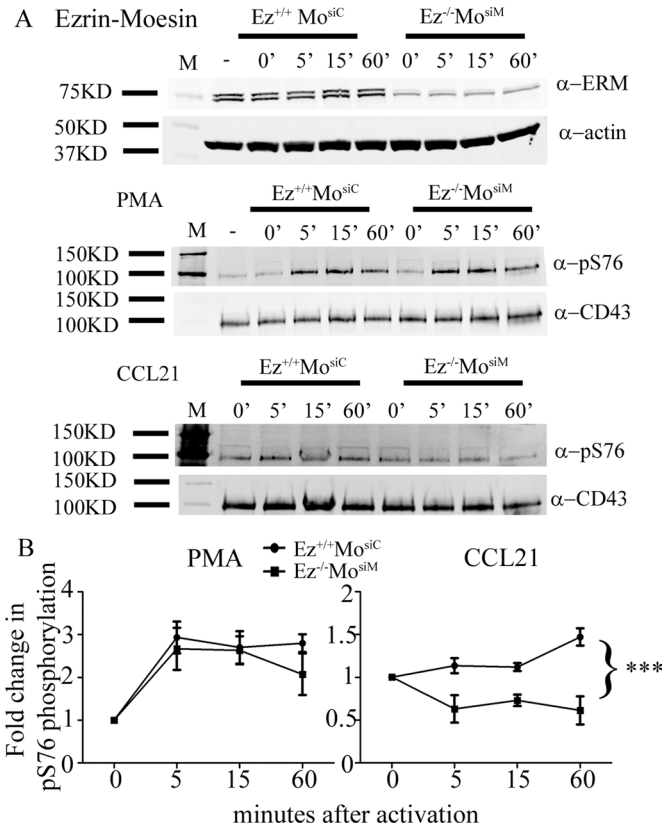


FIGURE 3: Ezrin/moesin double-deficient T-cells show defective CD43 S76 phosphorylation in response to chemokine signaling. (A and B) Ezrin$^{+/+}$ or ezrin$^{-/-}$ T-cells were transfected with siRNA duplexes to murine moesin or control siRNA as described in *Materials and Methods*. Ez$^{+/+}$Mo$^{siC}$ or Ez$^{-/-}$Mo$^{siM}$ cells were either unstimulated or stimulated with PMA or CCL21 as indicated, lysed, and analyzed by SDS–PAGE. (A) Samples were blotted for ERM and actin to determine the level of moesin knockdown and actin blotted as a loading control (top). Cells were then analyzed for CD43 S76 phosphorylation, and level of S76 phosphorylation and total CD43 are shown from one representative experiment (middle and bottom). (B) Quantitation of S76 phosphorylation in response to PMA and CCL21 normalized to total CD43 (average of three independent experiments). ***$p < 0.001$ using two-way analysis of variance comparing CCL21-induced pS76 phosphorylation in T-cells expressing Ez$^{+/+}$Mo$^{siC}$ and Ez$^{-/-}$Mo$^{siM}$ at all time points.

CCL21 compared with CD43-NGG within the same well (Figure 4A). CD43-NGG mutants also show defects in migration to the LFA-1 integrin ligand intercellular adhesion molecule 1 (ICAM-1) (unpublished data). These results suggest that a block in CD43 interaction with ERM proteins inhibits T-cell migration. We found no difference in the expression levels of CD62L, CCR7, or LFA-1 between CD43-FL and CD43-NGG T-cells (unpublished data), indicating that expression of these cell surface receptors is not responsible for any potential migration defect.

To confirm that the in vitro migration defects seen in the CD43-NGG mutants were recapitulated in vivo, we directly compared the ability of CD43-FL– and CD43-NGG–expressing T-cells to migrate to lymph nodes. Briefly, CD43-deficient T-cells are retrovirally transduced with either CD43-FL or CD43-NGG, labeled with different fluorescent dyes, and transferred into recipients (Mody *et al.*, 2007). After 16 h, blood, lymph nodes, and spleen were removed and the ratio of CD43-FL and CD43-NGG cells determined. The ratio in the blood and spleen remained ~1:1 (Figure 4B, 50% FL, 50% NGG), demonstrating that there is no selective advantage in survival or circulation of CD43-FL compared with CD43-NGG T-cells. In contrast, the lymph nodes showed preferential accumulation of CD43-FL compared with CD43-NGG T-cells, with 60% CD43-FL T-cells compared with 40% CD43-NGG T-cells (Figure 4B). These results show that CD43 association with ERM proteins is required for normal trafficking of T-cells to lymph nodes.

**Constitutive S76 phosphorylation enhances T-cell migration**

CD43 interaction with ERM proteins is important to regulate both S76 phosphorylation and T-cell trafficking. Because the lack of S76 phosphorylation leads to defects in migration, these results suggest that the defect in migration in the CD43-NGG T-cells is likely due to the inability of these cells to phosphorylate CD43 at S76. However, ERM proteins regulate many aspects of the actin cytoskeleton, and it is possible that a block in CD43–ERM interactions may affect T-cell migration independently of the effect on S76 phosphorylation. To test whether the migration defects in CD43-NGG T-cells are a result of the lack of S76 phosphorylation, we changed the serine residue at

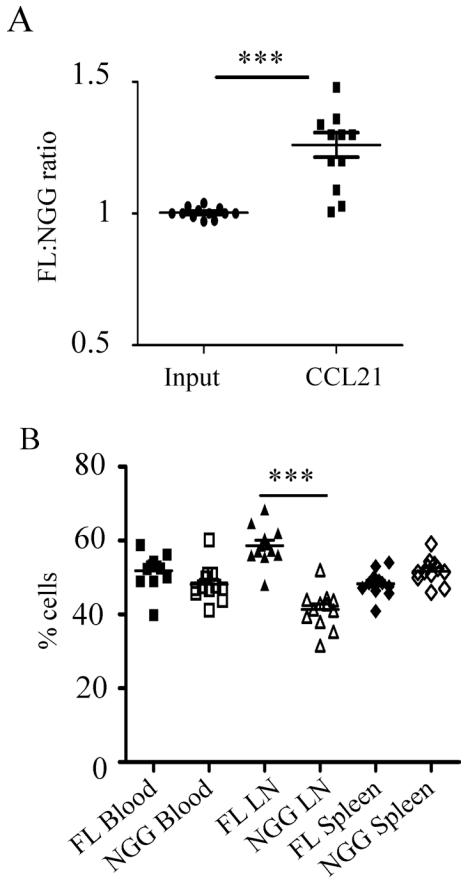


FIGURE 4: CD43 binding to ERM proteins regulates T-cell trafficking. (A and B) DO.CD43$^{-/-}$ T-cells were transduced with CD43-FL or CD43-NGG mutants, harvested, purified, and labeled as described in the *Materials and Methods*. (A) Equal numbers of FL and NGG T-cells were mixed and added to the top of a 3-μm Costar Transwell apparatus. The ratio of FL and NGG T-cell migration was assayed after addition of 300 ng/ml CCL21 to the bottom of the transwell for 1 h. ***$p < 0.001$. (B) CD43-FL T-cells were mixed with equal numbers of CD43-NGG T-cells and injected into recipients. Blood, spleen, and lymph nodes were harvested and analyzed for ratio of CD4$^+$ FL:NGG cells within the dyed population by flow cytometry. Horizontal bars represent the mean and the SD shown.

76 to an aspartic acid (S76D). The S to D mutation has been used in other proteins to mimic constitutive phosphorylation (Uff *et al.*, 1995). We find that the S76D mutant shows no effect on T-cell proliferation (Supplemental Figure 3A). We then tested the role of phosphorylation in migration using the transwell migration assay. CD43-S76D and CD43-FL cells were differentially fluorescently labeled, then added together, and allowed to migrate to CCL21. Interestingly, the S76D mutant slightly but significantly enhanced T-cell migration over that of CD43-FL T-cells, demonstrating that increasing S76 phosphorylation alone enhances T-cell migration (Figure 5A).

To test whether the migration defect in CD43-NGG T-cells is due to the block in S76 phosphorylation, we made the mutant that mimics constitutive phosphorylation at S76 (S76D) in combination with CD43-NGG, named the CD43-NGG-S76D. The CD43-NGG-S76D mutant mimics phosphorylation at S76 in the absence of CD43 binding to ERM proteins via the KRR. We introduced CD43-FL, CD43-NGG, and CD43-NGG-S76D mutants into CD43-deficient T-cells and compared the relative migration of each population in response to CCL21 and ICAM. CD43-NGG-S76D T-cells showed a slight (20%) but consistently significant increase in migration compared with CD43-NGG T-cells to CCL21 (Figure 5B). The same was found in response to ICAM (unpublished data). The trend was reproduced in the in vivo migration assay to lymph nodes, but the increase was not significant (unpublished data). The restoration of migration in CD43-NGG-S76D was partial, as CD43-NGG-S76D mutants still showed a defect in migration compared with CD43-FL T-cells (Supplemental Figure 4). These results show that constitutive S76 phosphorylation alone can mediate T-cell migration, even in the absence of the ERM binding site.

### S76 phosphorylation does not restore CD43 movement to uropods

While CD43 binding to ERM proteins regulates S76 phosphorylation, previous evidence suggested that the domain containing S76 within CD43 may also contribute to ERM binding (Yonemura *et al.*, 1998). CD43 localizes to both the distal pole and the uropod of migrating T-cells. The uropod is marked by a tail at one end of an elongated T-cell, often raised up from the body of the T-cell, projecting it slightly out of the plane of focus (Figure 6A, DIC). The uropod is clearly demarcated by the presence of the microtubule-organizing center, labeled as a concentrated point of tubulin staining (Figure 6A, tubulin). We and others have previously shown that CD43 localization to the DPC and the uropod is dependent on CD43 interaction with ERM proteins. To test the possibility that S76 phosphorylation may affect ERM association, we used CD43 localization to the uropod as a marker of ERM binding.

CD43-FL is efficiently moved to the uropod of migrating T-cells while CD43-NGG mutants show almost no movement to the uropod at all (see Figure 6, A and B). As the absolute number of cells showing CD43 localization to the uropod changes from experiment to experiment, we normalized the level of localization in CD43-FL–expressing T-cells to 100% and compared the level of localization in the CD43 mutants to CD43-FL. When the number of T-cells showing CD43 localized to the uropod in CD43-FL–expressing T-cells is compared with CD43-NGG mutants, fewer than 6% of CD43-NGG mutants show CD43 at the uropod (Figure 6B). The S76D mutant alone

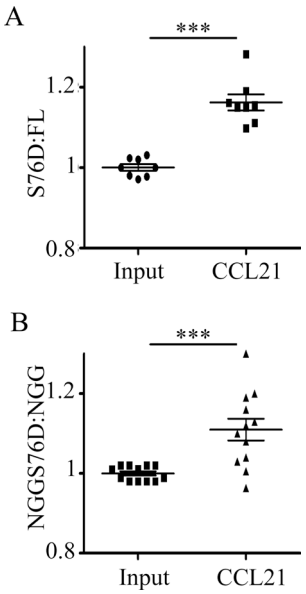


FIGURE 5: Constitutive phosphorylation at S76 partially reconstitutes T-cell migration in the absence of ERM protein binding. DO.CD43$^{-/-}$ T-cells were transduced with CD43-FL, CD43-S76D, CD43-NGGS76D, or CD43-NGG mutants; harvested; and labeled. (A) CD43-S76D– and CD43-FL– or (B) CD43-NGGS76D– and CD43-NGG–expressing T-cells were added to a 3-μm Costar Transwell apparatus and migration was assayed as described in Figure 3. ***$p < 0.001$.

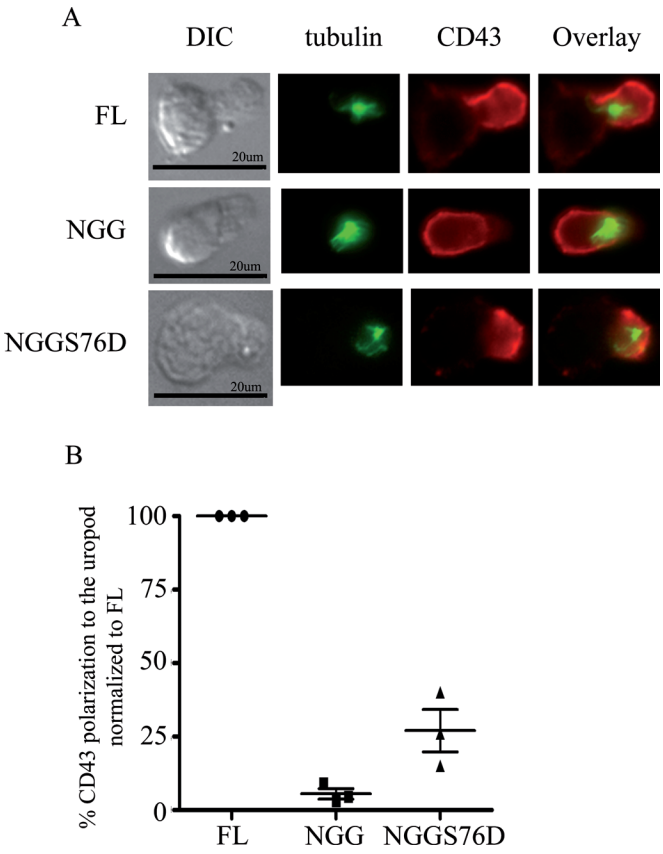


FIGURE 6: Constitutive phosphorylation at S76 does not reconstitute CD43 movement to the T-cell uropod. DO.CD43$^{-/-}$ T-cells were transduced with CD43-FL, CD43-NGGS76D, or CD43-NGG mutants; harvested; and labeled. Purified retrovirally transduced T-cells were treated with SDF1α and processed for immunofluorescence. Cells were scored for CD43 localization to the uropod. (A) Representative T-cells expressing CD43-FL, CD43-NGG, and CD43-NGGS76D mutants stained for tubulin and CD43. Scale bar indicates 20 μm. (B) Average of the quantitation of CD43 localized to the uropod in at least 50 cells in three independent experiments. Percentage of localization was normalized to CD43-FL as 100%.

does not change CD43 movement to the uropod or distal pole alone compared with wild-type CD43 (Supplemental Figure 3, B and C). To ask whether S76 phosphorylation can localize CD43 in the absence of KRR-mediated ERM binding, we assessed whether the S76D mutation in conjunction with the NGG mutant (CD43-NGG-S76D) would enhance CD43 localization to the uropod of migrating T-cells. While some CD43-NGG-S76D mutants showed CD43 localization to the uropod similar to CD43-FL (Figure 6A), quantitation of CD43 movement demonstrated that only ~25% of CD43-NGG-S76D–expressing T-cells showed CD43 localization at the uropod compared with CD43-FL (Figure 6B). The difference between CD43-NGG and CD43-NGG-S76D localization to the uropod approached but did not reach significance ($p = 0.06$). The vast majority of CD43-NGG-S76D mutants showed no specific CD43 localization to the uropod. These results show that CD43 phosphorylation cannot fully restore CD43 localization to the uropod in the absence of the KRR sequence.

### CD43 phosphorylation can restore CD43 localization to the DPC

In addition to localization to the uropod, CD43 also localizes to the DPC upon T-cell activation. The signals driving CD43 localization to the uropod are different from those that drive CD43 to the DPC as the uropod is formed by chemokine signaling while the DPC is formed after TCR activation. However, ERM proteins have been demonstrated to be important for CD43 localization to both structures (Allenspach *et al.*, 2001). To assess the role of CD43 phosphorylation on CD43 localization to the DPC, we took DO.CD43$^{-/-}$ T-cells transduced with CD43-FL and assessed CD43 movement to the distal pole upon activation by an A20 B-cell loaded with the cognate ovalbumin (OVA) peptide. Similar to CD43 localization to the uropod, in CD43-FL–transduced T-cells, CD43-FL moved efficiently to the DPC while CD43-NGG–expressing T-cells cannot redistribute CD43 to the distal pole (Figure 7A). As a control and shown previously, CD43 mutants do not perturb protein kinase Cθ (PKCθ) localization to the synapse, demonstrating that signaling to the synapse is intact in CD43-NGG and CD43-NGG-S76D mutants (Tong *et al.*, 2004 and Figure 7A). To quantitate the level of reconstitution of CD43 localization, we again normalized the level of CD43 localization to that seen in CD43-FL, as the absolute number of cells with CD43 localization differed from experiment to experiment.

Interestingly, CD43 movement to the distal pole is partially restored in CD43-NGG-S76D T-cells. Over 70% of the CD43-NGG-S76D–expressing mutants showed CD43 at the DPC when normalized to the number of CD43-FL T-cells with CD43 at the DPC (Figure 7B). This is in contrast to the CD43-NGG mutants, in which fewer than 25% of the CD43-NGG mutants show CD43 localization to the DPC (Figure 7B). These results show that CD43 S76 phosphorylation can mediate CD43 movement to the DPC in the absence of the KRR.

### CD43 phosphorylation does not directly enhance ERM binding

We have shown that constitutive CD43 phosphorylation partially restores CD43 movement to the DPC in the absence of the KRR, raising the possibility that S76 phosphorylation may confer ERM binding independently of the KRR. To ask whether CD43 phosphorylation directly mediates CD43 binding to ERM proteins, we tested the binding of the CD43-NGG-S76D mutant to ERM proteins. We transfected 293T cells with the green fluorescent protein (GFP)–tagged N-terminal FERM domain of ezrin responsible for CD43 binding. We then immunoprecipitated Ez-GFP with glutathione S-

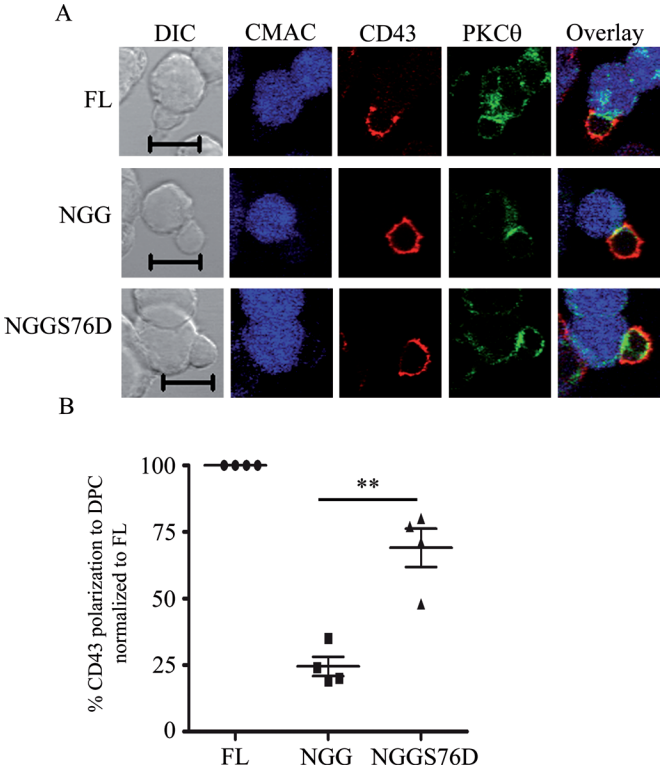


FIGURE 7: Constitutive phosphorylation at S76 partially reconstitutes CD43 movement to the distal pole complex. DO.CD43$^{-/-}$ T-cells were transduced with CD43-FL, CD43-NGGS76D, or CD43-NGG mutants; harvested; and labeled. Purified retrovirally transduced T-cells were conjugated to OVA peptide–loaded, CellTracker™ Blue (7-amino-4-chloromethylcoumarin) (CMAC)–stained A20 B-cells and processed for immunofluorescence. (A) Representative T-cell–B-cell conjugates are shown with the B-cell labeled with CMAC in blue, CD43 in red, and PKCθ in green. Scale bar indicates 10 μm. (B) Cells were scored for CD43 localization to the DPC as previously described. Average of the quantitation of CD43 localized to the DPC in at least 50 T–B-cell conjugates in four independent experiments. Percent localization was normalized to CD43-FL as 100%. **$p < 0.01$.

transferase (GST) proteins fused to the ICD of CD43. GST-CD43 pulled down Ez-GFP while the NGG mutant of the ICD did not, recapitulating published results that the KRR is important in ERM binding (Figure 8, A and B). In agreement with previously published data, we find that the NGG mutation significantly blocked CD43 binding to ezrin (Figure 8B). Interestingly, contrary to our hypothesis, we found that GST-CD43-NGG-S76D did not enhance binding to ERM proteins above that seen with GST-CD43-NGG. In fact, the S76D mutation alone abolished binding to Ez-GFP, blocking ezrin binding to the same extent seen with NGG (Figure 8B), suggesting that phosphorylation alone inhibits CD43–ERM association. We found the same results using in vitro phosphorylated GST-CD43 and GST-CD43-NGG mutants, suggesting that these results are not due to a structural artifact of the S to D mutation (unpublished data). These data demonstrate that phosphorylation at S76 does not enhance CD43 interaction with ERM proteins to mediate CD43 movement and T-cell migration.

### PKCθ can mediate CD43 S76 phosphorylation

To further investigate the signaling components that regulate T-cell trafficking via S76 phosphorylation, we sought to identify the kinase that may participate in phosphorylating CD43 at S76. We began our

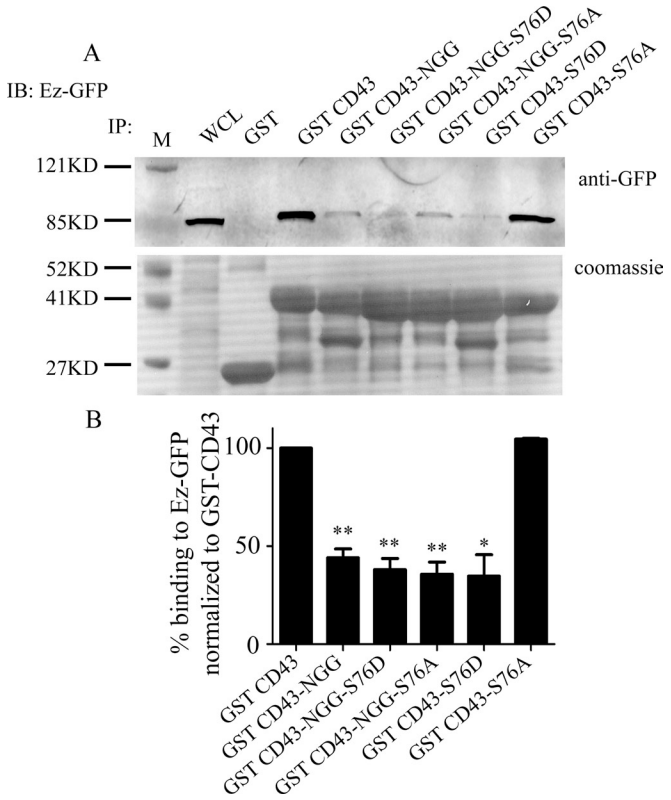


FIGURE 8: CD43 phosphorylation does not enhance ERM binding. (A and B) 293T cells transfected with Ez-FERM-GFP were lysed and lysates incubated with the indicated GST CD43 fusion proteins confirmed for equal loading by Coomassie stain. Immunoprecipitates were processed by SDS–PAGE and blotted with anti-GFP to detect ezrin. (B) Quantitation of ezrin binding phosphorylation shown in (A) normalized to GST signal in three independent experiments. *p < 0.05; **p < 0.01.

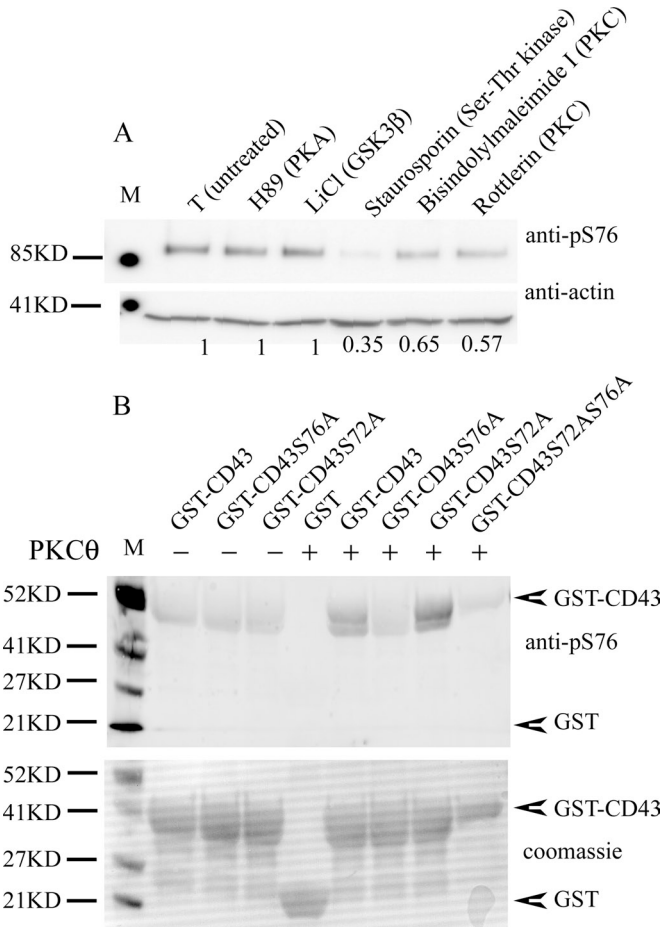


FIGURE 9: PKCθ can phosphorylate CD43 at S76. (A) T lymphoblasts were incubated in the presence of indicated inhibitors of specified kinases (in parentheses) for 2 h, cells were lysed, and lysates were analyzed on SDS–PAGE and blotted for phospho-S76 and actin. (B) GST, GST-CD43, and GST-CD43 mutant fusion constructs were incubated with or without purified PKCθ, analyzed by SDS–PAGE, detected for anti–phospho-S76, and stained with Coomassie stain.

analysis using broad-based kinase inhibitors to narrow our range of possible candidates. Computer-aided programs based on homology to known substrates identified the PKC family, as well as protein kinase A (PKA) and glycogen synthase kinase 3β (GSK-3β), as kinases that may phosphorylate S76. We compared the inhibition of S76 phosphorylation in the presence of inhibitors of PKA (H-89), PKC family members (bisindolylmaleimide I and rottlerin), and GSK-3β (LiCl) as well as a pan-serine-threonine kinase inhibitor (staurosporin). Staurosporin inhibits S76 phosphorylation almost completely, while inhibition of PKA and GSK-3β had no effect on S76 phosphorylation (Figure 9A). In contrast, inhibition of PKC family members by both bisindolylmaleimide I and rottlerin inhibited S76 phosphorylation by ~50%. These data together with our finding that PMA can enhance S76 phosphorylation narrowed the candidate kinase to a member of the PKC family.

PKC family members are categorized into several subgroups based on structure and responsiveness to stimuli: Classical (α, βI and βII, and γ) are activated by PMA and calcium; novel (δ, ε, θ, and η) are activated by PMA but not calcium; and atypical (ζ) are not activated by either PMA or calcium (Spitaler and Cantrell, 2004). S76 phosphorylation is increased by PMA (Figure 1) but not by calcium ionophore (unpublished data), suggesting that the candidate is likely to be in the novel subfamily. PKCθ is a novel PKC that is well characterized in T-cells, and thus we asked whether PKCθ was capable of phosphorylating CD43 in vitro. We generated GST fusion constructs containing the cytoplasmic tail of CD43 (GST-CD43) and added purified PKCθ. PKCθ phosphorylated GST-CD43 specifically at S76 as detected by anti–phospho-S76 (Figure 9B). There was no phosphorylation of GST alone without CD43. The S76 to A (GST-CD43-S76A) showed only background levels of staining by anti-pS76 antibody. As a control, we mutated another phosphorylation site identified in the CD43 cytoplasmic tail, S72 to A, which should not perturb S76 phosphorylation. The GST-CD43-S72A mutant shows the same level of S76 phosphorylation compared with GST-CD43. These results show that PKCθ can phosphorylate CD43 at S76.

## DISCUSSION

T-cell migration to lymph nodes is dependent on CD62L, CCR7, and LFA-1 on T-cells interacting with ligands on endothelial cells. We recently demonstrated a role for the cell surface mucin CD43 in regulating T-cell migration via the novel cytoplasmic serine phosphorylation site S76. We now find that S76 phosphorylation is responsive to migration cues and depends on CD43 association with ERM proteins. Phosphorylation at S76 alone can increase T-cell migration and CD43 localization to the DPC, but S76 phosphorylation-mediated migration and localization does not occur through enhanced ERM binding. Thus although CD43 phosphorylation depends on ERM binding, once phosphorylated, S76 phosphorylation

can regulate T-cell migration and CD43 localization independent of ERM binding.

ERM proteins can regulate T-cell motility through several different mechanisms. ERM proteins control microvilli formation and regulate microvilli length in multiple cell types including T-cells (Yonemura *et al.*, 1999; Brown *et al.*, 2003). ERM proteins also localize to the uropod and have been implicated in uropod formation, although whether they are required for uropod formation is still unclear (Lee *et al.*, 2004). Additionally, ERM proteins directly interact with several proteins that can regulate T-cell motility, including CD44 and CD62L, the selectin responsible for lymph node entry of T-cells (Ivetic *et al.*, 2002; Legg *et al.*, 2002). We now show that ERM proteins also regulate T-cell trafficking through CD43 S76 phosphorylation. CD43 regulation of T-cell motility depends on S76 phosphorylation, and we find that abrogation of CD43–ERM association either by CD43 mutation or ERM deficiency decreases S76 phosphorylation, which results in decreased T-cell trafficking both in vitro and in vivo.

The juxtamembrane KRR sequence within CD43 is necessary for CD43 binding to ERM proteins, as mutation of this sequence abolishes binding (Figure 8 and Yonemura *et al.*, 1998). However, an additional region between amino acids 62 and 78 of CD43 ICD was also shown to participate in ERM binding, and crystal structure evidence shows that amino acids adjacent to the KRR sequence also participate in CD43 binding to ERM proteins (Serrador *et al.*, 1998; Yonemura *et al.*, 1998; Takai *et al.*, 2008). Furthermore, phosphorylation of other ERM binding partners, CD44 and ICAM-3, can modulate ERM interaction (Legg *et al.*, 2002; Serrador *et al.*, 2002). These results led us to hypothesize that S76 phosphorylation may also regulate CD43–ERM association. Our findings show that phosphorylation at S76 decreased ERM binding even in the presence of the KRR sequence. The interaction between ICAM-3 and ERM proteins has also been shown to depend on serine residues (Serrador *et al.*, 2002). Similar to our findings, mutation of serines in CD44 and ICAM-3 to aspartic acid also inhibits CD44 and ICAM-3 interaction with ERMs. However, CD43 and CD44 differ from ICAM-3 in that while the S to A mutation blocks ICAM-3–ERM binding, S to A mutation alone in CD43 and CD44 does not affect ERM association (Figure 8 and Serrador *et al.*, 2002). Thus while serine residues and phosphorylation in CD43, CD44, and ICAM-3 all affect ERM binding, the specific effect of phosphorylation on ERM association differs between each downstream binding partner.

Interestingly, we find that while CD43 phosphorylation at S76 does not increase ERM association, constitutive phosphorylation in the absence of ERM binding partially restores both T-cell trafficking and CD43 movement to the DPC. This is surprising for two reasons. First, our previous results had shown that CD43 movement was dependent on ERM association (Tong *et al.*, 2004). Second, phosphorylation at S76 did not change CD43 localization to the DPC (Mody *et al.*, 2007). These data suggest that while ERM association by KRR is sufficient to mediate CD43 localization to the DPC, phosphorylation represents a mechanism to regulate CD43 movement and T-cell migration independent of ERM proteins. Our results also show that while CD43 phosphorylation is not required for CD43 movement to the DPC, the S76D mutation reveals an alternative ERM-independent pathway that can drive CD43 movement downstream of TCR signaling. It is also clear that CD43 binding to ERM proteins regulates T-cell migration independently of its effects on migration, as phosphorylation at S76 in the absence of ERM binding only partially restores T-cell migration. Our data demonstrate that two independent regulatory mechanisms within CD43 can control CD43 movement and effects on T-cell migration, the ERM binding domain (KRR) and the S76 phosphorylation site.

Our studies identify PKCθ as the likely kinase responsible for S76 phosphorylation. The PKC proteins are important signaling molecules downstream of extracellular receptors leading to changes in cellular proliferation, cytoskeletal changes, and differentiation (Spitaler and Cantrell, 2004). In T-cells, PKCθ is an important signaling molecule downstream of TCR leading to T-cell survival and differentiation through activation of NFκB, NFAT, and AP-1 (Tan and Parker, 2003). We hypothesize that ERM proteins may function to recruit kinases to CD43 mediating S76 phosphorylation. Interestingly, PKCθ was shown to bind to and phosphorylate moesin (Pietromonaco *et al.*, 1998). Our data do not preclude the possibility that other kinases may also phosphorylate and regulate S76 phosphorylation, such as LOK, which was recently shown to phosphorylate ERM proteins and mediate T-cell migration (Belkina *et al.*, 2009). It remains to be determined how precisely S76 phosphorylation regulates T-cell migration. Recent evidence demonstrates that the CD43 extracellular domain can be cleaved by γ-secretase and then the intracellular tail subsequently translocated to the nucleus to promote T-cell survival (Mambole *et al.*, 2008; Seo and Ziltener, 2009). It is possible that S76 phosphorylation may modulate CD43 translocation or its downstream effects in the nucleus. In addition, other groups have found that CD43 affects homotypic adhesion in T-cells through up-regulation of CD11a/CD18 (LFA-1) (Sanchez-Mateos *et al.*, 1995). While we see no changes in the expression of LFA-1 or other molecules that regulate T-cell trafficking, such as CD62L and CCR7 (unpublished data; Mody *et al.*, 2007), CD43 may affect the function of these molecules. We also find that CD43 S76 phosphorylation is responsive to both extracellular and intracellular cues that in turn regulate T-cell migration. The CD43 extracellular domain is highly glycosylated, and the glycosylation likely mediates CD43 interaction with several ligands that regulate T-cell migration, including E-selectin and galectin-1 (Matsumoto *et al.*, 2005; He and Baum, 2006). It is also possible that S76 phosphorylation may, in turn, regulate the binding of CD43 to its ligands and lead to changes in migration. Finally, S76 phosphorylation may provide a platform for yet unidentified novel interactions that transduce signals that result in changes to T-cell migration.

## MATERIALS AND METHODS

### Mice

TCR Tg DO11.10XCD43$^{-/-}$ (DO.CD43$^{-/-}$) were previously described (Tong *et al.*, 2004). All mice were bred and/or maintained in a specific pathogen-free condition in barrier facilities (Chicago, IL) and conform to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines. CD43-FL and CD43-NGG Tg mice were made in B6.CD43$^{-/-}$ mice by placing CD43-FL and CD43-NGG under the proximal *lck* promoter, human growth hormone gene polyadenylation site, and locus control region elements from the human CD2 gene (Wang *et al.*, 2001). Three independent founder lines of each were produced, and one of each was selected for maintenance based on similar cell surface expression of CD43.

### Reagents and antibodies

Affinity-purified α-CD28 (PV-1), α-CD3 (145-2C11), α-GFP, and α-CD43 (S11 and R2/60) were prepared in our laboratory. α-CD4, α-CD44, α-CD62L, α-CD45.1, α-CD45.2, α-CD90.1, and α-CD90.2 were from eBiosciences (San Diego, CA); α-actin was from Sigma Aldrich (St. Louis, MO); α-tubulin was from Thermo Fisher; and α-PKCθ was from Santa Cruz Biotechnology (Santa Cruz, CA). CCL21 was from Peprotech (Rocky Hill, NJ), and ICAM-Fc was from R&D Systems (Minneapolis, MN). For the Li-Cor Odyssey system, α-rabbit 680 conjugates were from Invitrogen, Molecular Probes (Carlsbad, CA), and α-rat 800 conjugates were from Rockland (Gilbertsville, PA).

### Generation of phospho-S76 antibody

Rabbit anti–phospho-S76-specific CD43 antibody was produced at Zymed Laboratories, Invitrogen (South San Francisco, CA) by immunizing rabbits with serine-phosphorylated CD43 peptide RQG-$_{P}$S$^{76}$-LVLEELK. We began counting at the intracellular region, so S76 is actually S347 from the starting methionine. The resulting antisera were positively affinity purified by binding the phosphorylated form of peptide coupled to resin and adding antisera to the column. To ensure no cross-reactivity with nonphosphorylated CD43, the eluted fraction comprising the positively purified antibodies was passed through a column bound with the equivalent unphosphorylated peptide. The flow-through was collected and tested for specific reactivity toward phosphorylated CD43 S76 (see *Results* and Supplemental Figure 1).

### Immunoblotting

Nylon-nonadherent lymph node T-cells or retrovirally transduced T-cells were treated with either 50 ng/ml PMA, 300 ng/ml CCL21, or 5 μg/ml α-CD43 (R2/60) for the indicated times and analyzed as previously described (Cannon *et al.*, 2008). Cells were lysed in lysis buffer (0.5% TX-100, 150 mM NaCl, 50 mM Tris [pH 7.6], 5 mM EDTA, 1 mM NaF, 1 mM $Na_3VO_4$, and protease inhibitors: 10 μg/ml aprotinin, 1 mM Pefabloc [Roche Applied Sciences, Mannheim, Germany], and 10 μg/ml leupeptin) before being analyzed by SDS–PAGE. For treatment with phosphatase, T-cells were lysed in lysis buffer as described previously, then treated with shrimp alkaline phosphatase for 30 min, and analyzed by SDS–PAGE. All Western blotting signal was detected with the Odyssey system (Li-Cor Biosciences, Lincoln, NE). For treatment with inhibitors, T-cells were treated with indicated inhibitors at the following concentrations for 2 h: 10 μM H89 (Biomol International, Plymouth Meeting, PA); 20 mM LiCl (Sigma Aldrich); 1 μM staurosporin, 1μM bisindolylmaleimide I, and 10 μM rottlerin (Calbiochem, Gibbstown, NJ).

### Construction of vectors

CD43 constructs were generated as previously described (Allenspach *et al.*, 2001; Mody *et al.*, 2007). The S76D mutation was generated using the following mutagenesis primers: 5′-CGGAAGTCTCGCCAGGGCGATTTAGTACTAGAAGAGC-3′ and 5′-GCTCTTCTAGTACTAAATCGCCCTGGCGAGACTTCCG-3′. For the GST constructs, the cytoplasmic tail of CD43 was cloned and fused to the pGEX3X GST expression vector starting at the first amino acid of the cytoplasmic tail (amino acid 271 from the start-site methionine). All CD43 mutants were made using the QuikChange Mutagenesis kit (Stratagene, La Jolla, CA).

### Preparation of GST constructs

GST constructs were transformed into the BL21 bacterial strain, grown and protein production induced with isopropyl β-D-1-thiogalactopyranoside, then bacteria lysed, and sonicated, and lysates were incubated with glutathione-Sepharose beads. Expression levels were tested by Coomassie staining before use in experiments.

### Preparation of ezrin/moesin double-deficient cells

Ezrin/moesin double-deficient T-cells were generated as previously described (Shaffer *et al.*, 2009). Ezrinflox/flox mice were crossed with CD4cre to generate conditional ezrin knockout mice. CD4$^{+}$ T-cells were purified from conditional knockout mice and suppressed with small interfering RNA (siRNA) against moesin. Controls were generated from littermate controls or B6 mice suppressed with control siRNA. Control and ezrin/moesin double-deficient T-cells were stimulated with PMA and CCL21, lysed, and analyzed as described above.

### GST-CD43 precipitation with ezrin-GFP

293T cells were transfected for 48 h with a truncated ezrin tagged with GFP as described in Allenspach *et al.* (2001) and lysed. Equal amounts of glutathione beads coupled to individual GST-CD43 mutants were added to cell lysates and incubated at 4°C for 2 h, and then GST precipitates were analyzed by SDS–PAGE and blotted with anti-GFP to detect the ezrin binding.

### In vivo migration

Competitive migration assays were performed as described with slight modification (Mody *et al.*, 2007). T-cells were labeled with either 5 μM carboxyfluorescein succinimidyl ester (CFSE) and 0.25 μM CFSE, or 5 μM CFSE and 4 μM PKH-26. Differentially dyed populations were mixed in equal numbers and $2 \times 10^7$ cells injected into recipient mice. Balb/c.Thy1.2 T-cells expressing CD43 mutant constructs were injected into Balb/c.Thy1.1 recipients. Input cells were identified by Thy1.2, CD4, CD43, and the dye. Organs were harvested and input cells identified by Ly5.2.

### Transwell migration assay

Approximately $1 \times 10^5$ T-cells were labeled with 5 μM CFSE or 0.25 μM CFSE, mixed, and added in a 1:1 ratio to the top of a Costar (Corning Acton, MA) 3.0-μm Transwell permeable support apparatus. For CCL21 conditions, 300 ng/ml CCL21 was added to the bottom of the transwell apparatus. For ICAM conditions, the transwell apparatus was coated with 6 μg/ml ICAM overnight, washed in phosphate-buffered saline, then blocked with 2.5% bovine serum albumin for 2 h, and washed, and cells were added to the top. At the end of the incubation period, transwell apparatuses and unmigrated cells were discarded, and migrated cells were analyzed and normalized to the input population using the LSRII (BD Biosciences, San Jose, CA).

### Immunofluorescence staining and microscopy

Immunofluorescence staining assessing CD43 localization was performed as previously described (Allenspach *et al.*, 2001).

### In vitro kinase assay

GST fusion constructs were prepared and incubated with purified PKCθ (Upstate Technologies, Millipore, Lake Placid, NY) using the manufacturer's protocol with nonradioactively labeled ATP. Phosphorylation at S76 was detected using the anti–phospho-S76 antibody.

### Statistical analysis

All statistics were done using a paired Student's two-tailed *t* test. Error bars represent standard error of the mean.

## ACKNOWLEDGMENTS

We acknowledge the flow cytometry and fluorescence microscopy facilities at the University of Chicago for their technical assistance. This study was supported by a research grant from the American Cancer Society–Illinois Division (J.L.C.) and National Institutes of Health grants R01 AI-044932 (A.I.S.) and R01 HL-71755 (N.O.D). J.L.C. was supported by a postdoctoral fellowship from the Irvington Institute Fellowship Program of the Cancer Research Institute and then by a Scientist Development Grant from the American Heart Association.

## REFERENCES

Allenspach EJ, Cullinan P, Tong J, Tang Q, Tesciuba AG, Cannon JL, Takahashi SM, Morgan R, Burkhardt JK, Sperling AI (2001). ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. Immunity 15, 739–750.

Andersson CX, Fernandez-Rodriguez J, Laos S, Sikut R, Sikut A, Baeckstrom D, Hansson GC (2004). CD43 has a functional NLS, interacts with β-catenin, and affects gene expression. Biochem Biophys Res Commun 316, 12–17.

Belkina NV, Liu Y, Hao JJ, Karasuyama H, Shaw S (2009). LOK is a major ERM kinase in resting lymphocytes and regulates cytoskeletal rearrangement through ERM phosphorylation. Proc Natl Acad Sci USA 106, 4707–4712.

Brown MJ, Nijhara R, Hallam JA, Gignac M, Yamada KM, Erlandsen SL, Delon J, Kruhlak M, Shaw S (2003). Chemokine stimulation of human peripheral blood T lymphocytes induces rapid dephosphorylation of ERM proteins, which facilitates loss of microvilli and polarization. Blood 102, 3890–3899.

Cannon JL, Collins A, Mody PD, Balachandran D, Henriksen KJ, Smith CE, Tong J, Clay BS, Miller SD, Sperling AI (2008). CD43 regulates Th2 differentiation and inflammation. J Immunol 180, 7385–7393.

Carlsson SR, Sasaki H, Fukuda M (1986). Structural variations of O-linked oligosaccharides present in leukosialin isolated from erythroid, myeloid, and T-lymphoid cell lines. J Biol Chem 261, 12787–12795.

Cullinan P, Sperling AI, Burkhardt JK (2002). The distal pole complex: a novel membrane domain distal to the immunological synapse. Immunol Rev 189, 111–122.

del Pozo MA, Sanchez-Mateos P, Nieto M, Sanchez-Madrid F (1995). Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. J Cell Biol 131, 495–508.

Delon J, Kaibuchi K, Germain RN (2001). Exclusion of CD43 from the immunological synapse is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. Immunity 15, 691–701.

Dustin ML (2009). The cellular context of T-cell signaling. Immunity 30, 482–492.

He J, Baum LG (2006). Endothelial cell expression of galectin-1 induced by prostate cancer cells inhibits T-cell transendothelial migration. Lab Invest 86, 578–590.

Ivetic A, Deka J, Ridley A, Ager A (2002). The cytoplasmic tail of L-selectin interacts with members of the ezrin-radixin-moesin (ERM) family of proteins: cell activation-dependent binding of Moesin but not Ezrin. J Biol Chem 277, 2321–2329.

Lee JH, Katakai T, Hara T, Gonda H, Sugai M, Shimizu A (2004). Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. J Cell Biol 167, 327–337.

Legg JW, Lewis CA, Parsons M, Ng T, Isacke CM (2002). A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility. Nat Cell Biol 4, 399–407.

Mambole A, Baruch D, Nusbaum P, Bigot S, Suzuki M, Lesavre P, Fukuda M, Halbwachs-Mecarelli L (2008). The cleavage of neutrophil leukosialin (CD43) by cathepsin G releases its extracellular domain and triggers its intramembrane proteolysis by presenilin/γ-secretase. J Biol Chem 283, 23627–23635.

Matsumoto M, Atarashi K, Umemoto E, Furukawa Y, Shigeta A, Miyasaka M, Hirata T (2005). CD43 functions as a ligand for E-selectin on activated T-cells. J Immunol 175, 8042–8050.

Mempel TR, Junt T, von Andrian UH (2006). Rulers over randomness: stroma cells guide lymphocyte migration in lymph nodes. Immunity 25, 867–869.

Mody PD, Cannon JL, Bandukwala HS, Blaine KM, Schilling AB, Swier K, Sperling AI (2007). Signaling through CD43 regulates CD4 T-cell trafficking. Blood 110, 2974–2982.

Pietromonaco SF, Simons PC, Altman A, Elias L (1998). Protein kinase C-θ phosphorylation of moesin in the actin-binding sequence. J Biol Chem 273, 7594–7603.

Piller V, Piller F, Fukuda M (1989). Phosphorylation of the major leukocyte surface sialoglycoprotein, leukosialin, is increased by phorbol 12-myristate 13-acetate. J Biol Chem 264, 18824–18831.

Sanchez-Mateos P, Campanero MR, del Pozo MA, Sanchez-Madrid F (1995). Regulatory role of CD43 leukosialin on integrin-mediated T-cell adhesion to endothelial and extracellular matrix ligands and its polar redistribution to a cellular uropod. Blood 86, 2228–2239.

Seo W, Ziltener HJ (2009). CD43 processing and nuclear translocation of CD43 cytoplasmic tail are required for cell homeostasis. Blood 114, 3567–3577.

Serrador JM *et al*. (1998). CD43 interacts with moesin and ezrin and regulates its redistribution to the uropods of T lymphocytes at the cell-cell contacts. Blood 91, 4632–4644.

Serrador JM, Vicente-Manzanares M, Calvo J, Barreiro O, Montoya MC, Schwartz-Albiez R, Furthmayr H, Lozano F, Sanchez-Madrid F (2002). A novel serine-rich motif in the intercellular adhesion molecule 3 is critical for its ezrin/radixin/moesin-directed subcellular targeting. J Biol Chem 277, 10400–10409.

Shaffer MH, Dupree RS, Zhu P, Saotome I, Schmidt RF, McClatchey AI, Freedman BD, Burkhardt JK (2009). Ezrin and moesin function together to promote T-cell activation. J Immunol 182, 1021–1032.

Sinclair LV, Finlay D, Feijoo C, Cornish GH, Gray A, Ager A, Okkenhaug K, Hagenbeek TJ, Spits H, Cantrell DA (2008). Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking. Nat Immunol 9, 513–521.

Sperling AI, Sedy JR, Manjunath N, Kupfer A, Ardman B, Burkhardt JK (1998). TCR signaling induces selective exclusion of CD43 from the T cell-antigen-presenting cell contact site. J Immunol 161, 6459–6462.

Spitaler M, Cantrell DA (2004). Protein kinase C and beyond. Nat Immunol 5, 785–790.

Takai Y, Kitano K, Terawaki S, Maesaki R, Hakoshima T (2008). Structural basis of the cytoplasmic tail of adhesion molecule CD43 and its binding to ERM proteins. J Mol Biol 381, 634–644.

Tan SL, Parker PJ (2003). Emerging and diverse roles of protein kinase C in immune cell signalling. Biochem J 376, 545–552.

Tong J, Allenspach EJ, Takahashi SM, Mody PD, Park C, Burkhardt JK, Sperling AI (2004). CD43 regulation of T-cell activation is not through steric inhibition of T-cell-APC interactions but through an intracellular mechanism. J Exp Med 199, 1277–1283.

Tsukita S, Yonemura S (1997). ERM proteins: head-to-tail regulation of actin-plasma membrane interaction. Trends Biochem Sci 22, 53–58.

Uff CR, Neame SJ, Isacke CM (1995). Hyaluronan binding by CD44 is regulated by a phosphorylation-independent mechanism. Eur J Immunol 25, 1883–1887.

von Andrian UH, Mempel TR (2003). Homing and cellular traffic in lymph nodes. Nat Rev Immunol 3, 867–878.

Wang J, Lo JC, Foster A, Yu P, Chen HM, Wang Y, Tamada K, Chen L, Fu YX (2001). The regulation of T-cell homeostasis and autoimmunity by T-cell-derived LIGHT. J Clin Invest 108, 1771–1780.

Yonemura S, Hirao M, Doi Y, Takahashi N, Kondo T, Tsukita S (1998). Ezrin/radixin/moesin (ERM) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of CD44, CD43, and ICAM-2. J Cell Biol 140, 885–895.

Yonemura S, Nagafuchi A, Sato N, Tsukita S (1993). Concentration of an integral membrane protein, CD43 (leukosialin, sialophorin), in the cleavage furrow through the interaction of its cytoplasmic domain with actin-based cytoskeletons. J Cell Biol 120, 437–449.

Yonemura S, Tsukita S, Tsukita S (1999). Direct involvement of ezrin/radixin/moesin (ERM)-binding membrane proteins in the organization of microvilli in collaboration with activated ERM proteins. J Cell Biol 145, 1497–1509.