Polar Redistribution of the Sialoglycoprotein CD43: Implications for T Cell Function

Nigel D. L. Savage, Stephanie L. Kimzey, Shannon K. Bromley, Kenneth G. Johnson, Michael L. Dustin and Jonathan M. Green

J Immunol 2002; 168:3740-3746; doi: 10.4049/jimmunol.168.8.3740
http://www.jimmunol.org/content/168/8/3740

References
This article cites 32 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/168/8/3740.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Polar Redistribution of the Sialoglycoprotein CD43: Implications for T Cell Function

Nigel D. L. Savage,* Stephanie L. Kimzey,* Shannon K. Bromley,† Kenneth G. Johnson,† Michael L. Dustin,‡ and Jonathan M. Green2§†

Contact between T cells and APCs results in the orchestrated segregation of molecules at the cell-cell interface and formation of a specialized structure termed the immunological synapse. This model predicts the topological seclusion of large molecules such as CD43 from the site of closest contact between the T cell and APC, allowing for the close apposition of cell membranes and effective TCR engagement. Similarly, during T cell migration segregation of CD43 to the uropod is thought to aid integrin adhesion at the leading edge of the cell by removing steric hindrance. We show in this work that CD43 distribution on T cells is regulated by a membrane proximal ezrin binding site and that failure to displace CD43 from the immunological synapse has no inhibitory effects on primary T cell activation. We also report that CD43 expression at the contact zone between T cells and matrix does not negatively regulate motility but may regulate LFA-1 de-adhesion. These results suggest that the steric barrier model of CD43 is inadequate and that alternative mechanisms account for the negative regulatory properties of CD43.


The interaction of T cells with APCs results in the formation of a specialized structure termed the immunological synapse, which consists of a TCR-rich central supramolecular activation complex (cSMAC) surrounded by a ring-shaped peripheral supramolecular activation complex (pSMAC) containing adhesion molecules such as LFA-1. This unique organization of surface proteins provides a molecular basis for both sustained adhesion and signaling necessary for successful T cell activation (1–5). The formation of the immunological synapse is thought to require the segregation of cell surface proteins based in part on size. Proteins that extend from the cell surface a distance similar to the TCR, including CD2 and CD28, are maintained in the cSMAC while larger proteins, including LFA-1 and CD22, are in the pSMAC (reviewed in Ref. 6). CD45 and CD43, which are among the most abundant and largest proteins expressed on T cells, are effectively excluded from the immunological synapse, suggesting that the presence of large molecules within the cSMACs and pSMACs could inhibit effective receptor-ligand interactions (7, 8).

CD43, in particular, has been implicated in negatively regulating T cell function, as well as the requirement for exclusion of CD43 from the pSMAC (reviewed in Ref. 9). The extracellular domain of CD43 is highly glycosylated and sialylated. This results in a negatively charged, rigid molecule, making CD43 an ideal candidate for nonspecific inhibition of protein-protein interactions at the cell surface (10, 11). CD43-deficient cells are more responsive to Ag and are hyperadhesive, presumably due to the reduced steric hindrance in the absence of the extracellular domain of CD43 (Refs. 12 and 13 and N. D. L. Savage and J. M. Green, unpublished data).

During locomotion, T cells form a distinct cellular structure at the trailing edge of the cell termed the uropod. Many proteins, including CD43, localize to the uropod. The leading edge of the cell has significantly increased sensitivity to Ag, despite similar densities of TCR (14). Relocalization of CD43 to the uropod is, therefore, assumed to support both T cell adhesion and activation by minimizing steric hindrance at the leading edge. However, this model of CD43 function, as well as the requirement for exclusion of CD43 from the T cell-APC contact zone, has not been formally tested.

The localization of CD43 to the cellular uropod in migrating lymphocytes has been thought to be dependent on the interaction of cytoplasmic domain of CD43 with the adapter protein ezrin, a member of the ezrin, radixin, and moesin (ERM) family (15, 16). ERM proteins link transmembrane proteins to actin by either direct or indirect interactions (17, 18). Thus, the anchoring of actin filaments to CD43 may be important in regulating cellular processes such as the establishment of cell polarity or cell motility, or in forming an initiating nucleus for the assembly of signal-transducing complexes (19).

We formally evaluated the requirement for CD43 redistribution for T cell activation, proliferation, and motility. Reconstitution of CD43-deficient primary T cells by retroviral gene transfer with specific mutants of CD43 demonstrates that the failure of CD43 to redistribute from the T cell-APC contact zone does not inhibit formation of an immunological synapse or proliferation in response to Ag. In addition, the redistribution of CD43 to the cellular uropod is not required for T cell locomotion. Taken together, our findings suggest a function of CD43 other than steric hindrance in inhibition of T cell function.

Materials and Methods

Mice

CD43-deficient mice were backcrossed to the DO11.10 TCR-transgenic background (provided by K. Murphy, Washington University, St. Louis, MO (20)) for seven generations. BALB/c mice were purchased from The

Departments of *Medicine and †Pathology and Immunology, Washington University School of Medicine, St. Louis, MO 63110; and ‡Program in Molecular Pathogenesis, Skirball Institute of Biomolecular Medicine, New York, NY 10016

Received for publication December 11, 2001. Accepted for publication February 11, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by National Institutes of Health Grant HL58444 (to J.M.G.). N.D.L.S. is a fellow of the Cancer Research Institute.

Address correspondence and reprint requests to Dr. Jonathan M. Green, Washington University School of Medicine, 660 South Euclid Avenue, Box 8052, St. Louis, MO 63110. E-mail address: greenj@mnotes.wustl.edu

Abbreviations used in this paper: cSMAC, central supramolecular activation complex; pSMAC, peripheral SMAC; ERM, ezrin, radixin, and moesin; Cy5, CyChrome 5; GFP, green fluorescent protein.
Jackson Laboratory (Bar Harbor, ME). Animals were maintained in specific pathogen-free housing at Washington University School of Medicine. All protocols have been approved by the Animal Studies Committee at Washington University School of Medicine.

**Antibodies**

Anti-CD43 hybridoma (S7, rat IgG2a) was provided by J. G. Frelinger (Rochester University, Rochester, NY). Anti-TCRβ hybridoma (H57-597, Armenian hamster IgG) was provided by P. M. Allen (Washington University). All other mAb were purchased from BD PharMingen (San Diego, CA). F(ab)₂ were generated using Pierce Immunopurine F(ab)₂ kit (Pierce, Rockford, IL). Abs and F(ab)₂ were conjugated to fluorochromes (Cy-Chrome 3, CyChorme 5 (Cy5), and Alexa 488; Molecular Probes, Eugene, OR).

**Constructs**

Full-length murine CD43 cDNA was cloned into the retroviral vector GFP-P2 (21) (provided by W. Sha, University of California, San Francisco, CA) or tailless human CD4RV (hCD4RV, provided by K. Murphy). All mutations were introduced by oligonucleotide site-directed mutagenesis using the Quickchange kit (Stratagene, La Jolla, CA). The CD43/CD102 chimera was made by overlap extension PCR (CD102 cDNA provided by T. Springer, Center for Blood Research, Boston, MA). Sequences were confirmed by automated fluorescent sequence analysis (Big Dye; Applied Biosystems, Lincoln, CA).

**Retroviral infections**

Retroviral vectors containing CD43 were transiently transfected into the Phoenix Eco packaging cell line (provided by G. Nolan, Stanford University, Palo Alto, CA) and T cells infected with retroviral supernatant as previously described (22). Expression of CD43 was confirmed in all experiments by flow cytometry. When indicated, T cells retrovirally infected with the hCD4RV constructs were enriched using immunomagnetic cell sorting and anti-human CD4 beads with an AutoMACS sorter (Miltenyi Biotec, Auburn, CA).

**APC-T cell interaction**

DO11.10 CD43⁻/⁻ T cells were infected with retrovirus containing CD43 with the indicated mutations. L cells transfected with I-A and ICAM-1 complete medium with 10⁻⁴ M OVA 323–339 peptide were added and prolifera-

**Proliferation assays**

DO11.10 CD43⁻/⁻ T cells were infected with retrovirus encoding for either full-length CD43 or mutant CD43 proteins and 2.5 × 10⁵ T cells cocultured with 1.25 × 10⁸ irradiated BALB/c splenocytes in complete medium. Graded doses of OVA 223–239 peptide were added and prolifera-

**Statistical analysis**

Statistical significance of the data obtained from motility studies and fluorescence intensity was determined by an unpaired two-tailed t test using Excel software (Microsoft, Seattle, WA).

**Results and Discussion**

A membrane proximal cytoplasmic domain (KRR) regulates localization of CD43

To determine the domain within CD43 that regulates its redistribution upon T cell polarization, we reconstituted primary T cells from CD43-deficient mice with mutant CD43 protein using retroviral gene transduction. Wild-type or mutant CD43 constructs (Fig. 1a) were cloned into the retroviral vector GFP-P2, which encodes for a bicistronic message allowing for expression of green fluorescent protein (GFP) and CD43. Flow cytometric analysis demonstrated that the expression of the retrovirally encoded CD43 protein was at similar levels to the endogenous protein, with the exception of the CD43Δcyto (Fig. 1b). The CD43Δcyto construct consistently had lower levels of expression, suggesting that the stability of the expressed protein may be decreased due to deletion of the cytoplasmic domain. The retrovirally expressed CD43 was recognized normally by a variety of anti-CD43 mAb (Fig. 1a and data not shown), some of which recognize carbohydrate-dependent epitopes, suggesting normal glycosylation of the retrovirally encoded CD43. In addition, as we used primary T cells derived from mice in which the CD43 gene has been deleted by homologous recombination, it is unlikely that there are defects in the glycosyltransferase enzymes that mediate posttranslational modification of CD43. Therefore, it is likely that the retrovirally expressed CD43 is glycosylated and sialylated in a manner similar to endogenous CD43.

To determine the localization of CD43, retrovirally infected T cell blasts from CD43-deficient mice were adhered to supported

**FIGURE 1.** Generation of CD43 mutants and identification of a membrane proximal site responsible for CD43 distribution. a, Diagram representing the intracellular domain of CD43 constructs used in these studies. Premature stop codons (CD43Δcyto, CD43 STOP1–4) and three amino acid substitutions (CD43NGG) as well as a chimeric molecule consisting of CD43 extracellular and transmembrane domains with the intracellular domain of CD102 (CD43/CD102) were generated by PCR and cloned into the bicistronic retroviral vectors GFP-P2 and hCD4RV. The darkened box depicts the mutated ERM binding site. The numerals represent the amino acid number. b, Flow cytometric analysis of CD43 expression on retrovirally infected T cells. T cells from CD43-deficient mice were infected with the indicated constructs and stained for CD43 expression with PE-conjugated S7 mAb. Retrovirally infected cells expressing GFP were gated on and presented is a histogram of the CD43 expression of these cells. Wild-type cells were mock infected and stained for endogenous CD43.

Supported planar lipid bilayers containing Cy5-conjugated GPI-ICAM-1 were prepared as previously described (1). Ab- and F(ab)₂-stained retrovirally infected T cells were injected into a heated FCS2 chamber (Bioptechs) and allowed to adhere, and data were acquired for 360 s using the Zeiss LSM 510 confocal microscope. Data analysis of trajectory and velocity of T cells was performed using National Institutes of Health ImageJ analysis software. Two-tailed t tests were performed to determine the statistical significance of any differences.
lipid bilayers containing GPI-anchored ICAM-1 as previously described (1). Examination by confocal microscopy revealed that deletion of the entire cytoplasmic domain prevented redistribution of CD43, whereas cells expressing the C-terminal deletion mutants CD43 STOP1–4 redistributed the receptor to the uropod (Fig. 2a). In these cells, the entire amount of surface CD43 is concentrated in the uropod, leading to a higher local density of CD43. Within the membrane proximal region, the amino acid sequence KRR, at position 276–278, has been demonstrated to mediate interaction of CD43 with the cytoskeletal adapter protein ezrin (24). Consistently, mutation of the KRR motif to NGG within the full-length cytoplasmic tail of CD43 abrogated the redistribution of CD43 to the uropod. False color image analysis of the fluorescence intensity demonstrated high expression of CD43 throughout the cell in the nonredistributing mutants (Fig. 2a). In both the CD43NGG- and CD43Δcyto-expressing cells, there is some suggestion of nonuniform clustering of CD43; however, the significance of this is unclear. Expression of a chimeric protein consisting of CD43 extracellular and transmembrane domain fused with the cytoplasmic portion of ICAM-2 (CD102), which also contains a membrane proximal ERM-binding site, restored the polar localization of the chimeric receptor. Analysis of the percentage of cells that form uropods revealed no difference in the ability of the cells to form a uropod based on the expression of mutant CD43. However, virtually all of the cells that did form uropods redistributed CD43, with the exception of the CD43Δcyto and CD43NGG constructs, in which essentially none of the cells redistributed CD43 (Table 1). The number of cells forming uropods in cells expressing the CD43

![Figure 2](http://www.jimmunol.org/)
STOP 1–4 constructs were similar to wild type (data not shown). Thus, the redistribution of CD43 on the cell surface is an active process requiring a specific interaction of the cytoplasmic domain of CD43 with intracellular proteins.

To determine whether cells expressing the nonredistributing CD43 mutants were capable of localizing other proteins to the uropod, endogenous CD102 was examined (25). Tight clustering of CD102 (Fig. 2b, blue staining) at the uropod indicates that the...
Presence of CD43 in the T cell-APC contact zones does not affect T cell proliferation

We evaluated whether expression of nonredistributing CD43 mutants would prevent the formation of an immunological synapse and/or inhibit the T cell proliferative response to Ag. CD43 constructs were expressed in DO11.10 TCR-transgenic CD43-deficient T cells by retroviral gene transfer. Infected cells were sorted by immunomagnetic selection and CD43 expression was confirmed by flow cytometry (data not shown). Sorted T cells were cocultured with ICAM-1- and I-A^d-transfected L cells previously loaded with OVA(323-339) peptide. T cell-APC contact and CD43 exclusion were monitored by confocal microscopy. T cells infected with virus encoding full-length CD43 (CD43FL) excluded CD43 from cell-cell contacts, as did CD43^+/+ controls (Fig. 3a and data not shown). Cells expressing CD43/CD102 also redistributed the chimeric protein to areas of no cellular contact, consistent with ezrin redistribution (Fig. 3a and data not shown). The CD43NGG and CD43Δcyto mutants failed to exclude CD43 from the site of contact (Fig. 3a) without affecting the ability of the T cell to generate sustained cSMACs. All cells expressing CD43FL or CD43/CD102 that formed a stable contact with the APC redistributed CD43, whereas all CD3NGG and CD43Δcyto cells failed to redistribute. Quantification of the intensity of CD43 staining at both the T cell:L cell contact and the region of the T cell not in contact with the L cell confirmed a failure to redistribute CD43 in the CD43Δcyto-expressing cells (Fig. 3b). In addition, there was no difference in the intensity of CD43 staining in the cSMAC as compared with the pSMAC. Interestingly, there was partial exclusion of CD43 from the contact region in cells expressing CD43NGG. Previous studies have demonstrated that there is a second ERM protein binding site in the tail of CD43 (24). This site is preserved in the CD43NGG mutant and may mediate the movement of this protein from the contact. Nonetheless, neither the CD43Δcyto nor the CD43NGG constructs were excluded to the same degree as the full-length CD43 or CD43/CD102 constructs, which were virtually completely redistributed away from the T cell:L cell contact. Both the CD43Δcyto- and CD43NGG-expressing cells formed a stable immunologic synapse, suggesting that, despite the large size and negative charge of the protein, redistribution away from the region of cell:cell contact is not required for formation of this structure. Interestingly, these observations are in contrast to data obtained by Wild et al. (26) which determined that extension of another protein, CD48, can inhibit T cell recognition of Ag, presumably by disrupting close apposition of cell membranes, thus disrupting long-term protein-ligand interaction, which would otherwise lead to cellular activation.

In addition to formation of a stable cSMAC, the proliferative response of cells expressing nonredistributing CD43 was comparable to cells expressing wild-type CD43 (Fig. 3c). Thus, the presence of CD43 at the site of cell-cell contact does not inhibit functional TCR engagement. Consistent with the finding that large molecules can be resident among smaller-sized proteins without necessarily disrupting formation of an immunologic synapse, CD45, another large molecule, was found to actively migrate into cSMACs during immunological synapse formation (23).

The ability of TCR engagement to occur in the absence of CD43 redistribution is supported by a recent series of papers examining the role of ERM proteins in the segregation of T cell surface proteins during T cell activation (27–29). Delon et al. (28) demonstrated that CD43 interacted with moesin and that dephosphorylation of moesin resulted in the release of CD43. The dephosphorylation occurred in response to TCR signaling and led to the release of CD43 from the cytoskeleton, which permitted an initial passive exclusion of the molecule from the contact. Re-anchoring of CD43 to the cytoskeleton was then required for complete exclusion. However, the initial release required engagement of the TCR, suggesting that effective TCR engagement occurs before redistribution of CD43. Furthermore, TCR-induced Ca^{2+} influx was normal in cells expressing a nonredistributing mutant of CD43. Similarly, Roumier et al. (27) found redistribution of ezrin to occur in response to TCR signaling and to depend upon Lck activity. Allenspach et al. (29) found redistribution of ezrin to occur in response to TCR signaling and to depend upon Lck activity. Interestingly, both groups demonstrate a reduction in IL-2 secretion by cells expressing nonredistributing mutants of CD43, whereas we find that proliferation is normal. Thus, it may be that while initial engagement is normal in the absence of CD43 redistribution, prolonged signaling is not maintained. However, our

![FIGURE 4. Presence of CD43 at cell-matrix interface is not an inhibitor of LFA-1/ICAM-1 interaction. a, Six confocal images of retrovirally infected T lymphocytes with CD43FL and CD43NGG constructs placed on ICAM-1-containing bilayers for 10 min to initiate uropod formation. Placing the movement of cells for a period of 6 min, we established that the trajectory between constructs was comparable and there was no difference observed between constructs tested. b, The velocity of the cells on ICAM-1 bilayers at 37°C (rate = distance/time) was calculated for each construct. There was no significant difference in the velocity of the cells expressing CD43FL, CD43Δcyto, or CD43NGG by two-tailed t test (p > 0.05).](http://www.jimmunol.org/Downloadedfrom)
data suggest that this defect is not sufficient to lead to a decrease in proliferative responses.

These data suggest that cell-cell interactions should not be regarded purely as flat and rigid membrane interactions but rather as highly active undulating processes in which differently sized molecules can be in relative proximity without sterically hindering one another. Alternatively, molecules like CD43 may display considerable flexibility at physiological ion concentrations, such that they are accommodated in 15-nm contact regions formed at the site of TCR:MHC interactions. The electron microscopy studies that show CD43 as a rigid rod were performed at low ionic strength, where the low dielectric constant reduces the shielding between sialic acids. The sialic acids then strongly repel each other, resulting in a fully extended conformation where the distance between sialic acids is maximal (10). At physiological ionic strengths the dielectric constant is higher and the repulsion between sialic acids is lower. Under these conditions CD43 should be much more compressible and may coexist with the TCR contacts where the relatively slow (seconds to minutes) contact formation process allows time for conformational changes in CD43 leading to interdigititation of CD43 chains in the 15-nm contact area (30). The negative role of CD43 in adhesive interactions with endothelial cells in flow conditions (31) may be more related to the very rapid kinetics of these interactions that do not allow time for the CD43 to move out of the way of interacting receptors.

These studies demonstrate that during a physiologically relevant interaction, i.e., T cell with an APC, the presence of CD43 in the cSMAC does not inhibit T cell proliferation. However, it remains a formal possibility that the kinetics of cell activation could be altered by the presence of CD43 in the cSMAC. Changes in the time course of effective TCR engagement would not be evident by measurement of proliferation. However, these data do establish that failure to exclude CD43 from the T cell-APC contact does not preclude effective T cell activation.

**Presence of CD43 in the adhesion contact zone does not inhibit T cell motility but affects de-adhesion**

Polarization of T cells occurs during cell migration, resulting in the redistribution of CD43 to the uropod. The leading edge, which is depleted in CD43, is the site at which initial adhesion receptor engagement occurs. The activity of both adhesion receptors and the TCR is increased in this area of the cell (14, 32). This has been thought to be due in part to the removal of large proteins such as CD43, which may sterically inhibit the interaction of these receptors with ligand. To test this hypothesis, primary T cells expressing either wild-type or mutant CD43 were placed on a supported planar lipid bilayer containing GPI-anchored ICAM-1. Cross-sectional reconstituted images (Z-stack) obtained by confocal microscopy confirmed the presence of CD43 at the cell-bilayer interface (Fig. 4). Time lapse analysis of migrating cells demonstrated that expression of nonredistributing mutant of CD43 did not impair T cell trajectory (Fig. 4a) and/or velocity (Fig. 4b) compared with redistributing constructs. Thus, the redistribution of wild-type CD43 to the uropod, while a consequence of the movement of ezrin to the uropod (16), which itself may or may not have functional consequences, is not required for effective engagement of adhesion receptors by their ligands.

In contrast to these observations, expression of the CD43NGG mutant did seem to affect LFA-1 disengagement (Fig. 5). Motile cells expressing CD43NGG had a striking phenotype of long residual strands of membrane trailing the cell that remained adherent to the bilayer which was not observed in CD43Δcyto-expressing cells. These stained heavily for CD43, and the persistent adherence of the strands did not inhibit migration velocity over the 6-min observation period. IRM images obtained in conjunction with Z-stack reconstitution of retrovirally infected cells revealed another striking observation. The uropods of cells expressing CD43NGG and CD43Δcyto were in full contact with the bilayer compared with endogenous CD43-, CD43FL-, and CD43/CD102-expressing cells, which clearly elevated uropods from the bilayer (Fig. 5, arrowheads in Z-stack and IRM panels).

Both the CD43Δcyto and the CD43NGG mutations resulted in contact of the uropod with the bilayer. However, the trailing strands were observed only in the CD43NGG mutation. Thus, the specific loss of the ezrin binding site in CD43, while keeping the remainder of the cytoplasmic interactions intact, resulted in this phenotype. This suggests that the binding of a distinct cytoplasmic protein to CD43 in the absence of ezrin binding to the proximal tail is responsible or, alternatively, that the introduction of the NGG mutation resulted in new protein-protein interaction with CD43.

The precise function of CD43 remains controversial (9). The most consistent observation has been that CD43-deficient cells have increased adhesion. This has been demonstrated both in vitro and in vivo (12, 13, 31, 33). The inhibitory function of CD43 on cell adhesion has been presumed to be mediated by the structural features of the extracellular domain, resulting in the steric hindrance of receptor ligand interactions. The data presented in this work suggest that this mechanism is not a tenable explanation. Disruptions in cytoskeletal associations or other signaling pathways could result in the alterations in the regulation of cell adhesion molecule activity. These and other possibilities remain to be explored as we move beyond the steric barrier model of CD43 function.

**Acknowledgments**

We thank Blair Ardman for providing CD43-deficient mice, Terry Woodford-Thomas, Andy Chan, and Andrey Shaw for access to the Zeiss confocal microscope, Arup Chakraborty for discussions on polymer dynamics, and Robert Arch for many discussions and for critical review of the manuscript.

**References**


