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CD43 Functions as a Ligand for E-Selectin on Activated T Cells

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E-selectin, an inducible cell adhesion molecule expressed on endothelial cells, mediates the rolling on endothelium of leukocytes expressing E-selectin ligands, such as neutrophils and activated T cells. Although previous studies using mice lacking P-selectin glycoprotein ligand-1 (PSGL-1) have indicated that PSGL-1 on Th1 cells functions as an E-selectin ligand, the molecular nature of E-selectin ligands other than PSGL-1 remains unknown. In this study, we show that a 130-kDa glycoprotein was precipitated by an E-selectin-IgG chimera from mouse Th1 cells. This protein was cleaved by O-sialoglycoprotein endopeptidase and required sialic acid for E-selectin binding. The mAb IB11, which recognizes the 130-kDa glycoform of CD43, recognized the 130-kDa band in the E-selectin-IgG precipitate. In addition, immunoprecipitation of the E-selectin-IgG precipitate with IB11 depleted the 130-kDa protein, further confirming its identity as CD43. CD43 was also precipitated with E-selectin-IgG from cultured human T cells. E-selectin-dependent cell rolling on CD43 was observed under flow conditions using a CD43-IgG chimera generated in Chinese hamster ovary cells expressing α,1,3-fucosyltransferase VII and a core 2 β,1,6-N-acetylgalactosaminyltransferase. These results suggest that CD43, when modified by a specific set of glycosyltransferases, can function as an E-selectin ligand and therefore potentially mediate activated T cell migration into inflamed sites. The Journal of Immunology, 2005, 175: 8042–8050.

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However, whether these glycoprotein ligands support physiologically relevant interactions with E-selectin is not yet certain.

Studies using Ab blockade as well as gene targeting have demonstrated that E-selectin, together with P-selectin, plays a critical role in the migration of neutrophils as well as certain subsets of T cells into sites of inflammation (15–17). In a model of contact hypersensitivity, E- and P-selectin cooperatively mediate Th1 cell migration into the skin during the effenter phase (18). Using PSGL-1-deficient (PSGL-1−/−) mice, we have shown that PSGL-1 functions as the predominant P-selectin ligand as well as one of the E-selectin ligands on Th1 cells in this model (6). The identity of physiological E-selectin ligands other than PSGL-1 on Th1 cells remains unknown.

CD43 is a cell-surface sialoglycoprotein expressed by most hematopoietic cells, including T cells (19). The extracellular domain of both human and mouse CD43 contains >80 serine or threonine residues, most of which are glycosylated by O-linked glycans that are heavily sialylated, and has a rod-like structure that is predicted to extend 45 nm from the lipid bilayer (20). Its extended structure and negatively charged sialic acid residues may confer an antiahesive function to this molecule. Indeed, CD43-deficient (CD43−/−) T cells show a marked increase in their in vitro proliferative response and homotypic adhesion (21) and increased tethering to L-selectin ligands in vivo and in vitro (22), indicating that CD43 negatively regulates T cell activation and adhesion. CD43−/− neutrophils also show enhanced rolling and adhesion both in vivo and in vitro (23). Paradoxically, however, CD43 deficiency reduces the recruitment of leukocytes into the peritoneal cavity (23), and the administration of an anti-CD43 mAb inhibits T cell migration into secondary lymphoid organs (24), implicating CD43 in a proadhesive process. How CD43 mediates apparently opposing functions remains unclear.

Two major glycoforms of human CD43 of 115 and 135 kDa have been identified (19). Similarly, two major glycoforms of 115 and 130 kDa have been identified for mouse CD43 (25). The 115-kDa glycoform is expressed on all T cells, whereas the larger glycoform is expressed predominantly on activated T cells. The O-glycans attached to the larger glycoform consist of a branched hexasaccharide core generated by C2GnT, which is up-regulated during T cell activation. In contrast, the O-glycans attached to the 115-kDa glycoform consist of a tetrasaccharide core.

In this study, we report that the 130-kDa glycoform of CD43 was precipitated from mouse Th1 cells using an E-selectin-IgG chimera as an affinity matrix. CD43 was also precipitated with E-selectin-IgG from human T lymphoblasts. Furthermore, using a CD43-IgG chimera, we show that CD43, when appropriately modified by a set of glycosyltransferases, supported E-selectin-dependent cell rolling under flow conditions. Taken together, our findings suggest that CD43 can function as a ligand for E-selectin on activated T cells and may potentially mediate activated T cell migration into sites of inflammation.

Materials and Methods

Mice

C57BL/6J mice were purchased from CLEA Japan. PSGL-1−/− mice on a C57BL/6J background were provided by Dr. B. Furie (Harvard Medical School, Boston, MA). All mice used were 6–12 wk of age. The mice were housed at the Institute of Experimental Animal Sciences at Osaka University Medical School (Osaka, Japan). All studies and procedures were approved by the Ethics Review Committee for Animal Experimentation of the Osaka University Graduate School of Medicine.

Chimeric proteins

To generate the mouse CD43-IgG construct, a fragment corresponding to the extracellular domain was amplified by PCR and ligated into the CD5 leader-IgG1 vector, provided by Dr. B. Seed (Massachusetts General Hospital, Boston, MA), at the Nhel and BamHI sites. To generate the human CD43-IgG construct, oligonucleotides that carry Apal and EcoRV sites were first inserted into the CD5 leader-IgG1 vector, and then a fragment corresponding to the extracellular domain of human CD43 was ligated at the Apal and EcoRV sites. The mouse and human PSGL-1-IgG constructs containing the entire extracellular domain were generated as previously described (26). The constructs were introduced by transient transfection into Chinese hamster ovary (CHO) cells stably expressing both human Fuc-T-VII and C2GnT (CD57I cells) (27), provided by Dr. R. McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK), with ESCORT V (Sigma-Aldrich). The chimeric proteins were purified from culture supernatants using protein A-Sepharose as previously described (26). The expression plasmids for mouse E- and P-selectin-IgM chimeric proteins were provided by Dr. J. Lowe (University of Michigan Medical School, Ann Arbor, MI). COS-7 cells were transfected with the plasmids using DEAE-dextran. Mouse E- and P-selectin-IgM chimeric proteins were prepared as described previously (6).

Preparation of mouse Th1 cells

Splenic CD4+ T cells were isolated by autoMACS (Miltenyi Biotec) using biotinylated anti-CD45RB (M1/70; BD Biosciences), biotinylated anti-B220 (RA3-6B2; BD Biosciences), and biotinylated anti-CD8α (53-6.7; Southern Biotechnology Associates), followed by Streptavidin MicroBeads (Miltenyi Biotec). Alternatively, they were isolated with a BD IMag CD4 T Lymphocyte Enrichment set (BD Biosciences). The enriched population was >90% positive for CD4 staining. Purified CD4+ T cells were cultured on 6-cm tissue-culture dishes coated with 10 μg/ml anti-CD3ε (145-2C11; BD Biosciences) and 10 μg/ml anti-CD28 (37.51; BD Biosciences) for 2 days in the presence of 4 ng/ml IL-2 (R&D Systems), 8 ng/ml IL-12 (R&D Systems), and 0.2 μg/ml anti-IL-4 (11B11; BD Biosciences). The cells were then transferred to uncoated dishes and cultured for an additional 4 days.

Preparation of human T lymphoblasts

Human blood from healthy adult donors was collected into tubes containing EDTA, and the mononuclear fraction was isolated following centrifugation through Ficoll-Paque PLUS (Amersham Biosciences). Mononuclear cells were cultured in 24-well plates coated with 10 μg/ml anti-CD3 (UCHT1; ebioscience) for 2 days in the presence of 5 ng/ml IL-2 (R&D Systems) in X-VIVO 15 medium (BioWhittaker). The cells were then transferred to uncoated dishes and expanded in X-VIVO 15 medium containing 5 ng/ml IL-2 for an additional 6 days. Human blood samples obtained in accordance with protocols approved by the Ethics Review Committee for Human Studies of the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

Flow cytometry

All mAbs used for the flow cytometric analyses were purchased from BD Biosciences. They included anti-CD4-FITC (RM4-5) and two anti-CD43 mAbs, 1B11-PE and S7-FITC. Cells were then transferred to uncoated dishes for 30 min on ice, washed, and analyzed on an EPICS XL flow cytometer (Beckman Coulter) or a FACSCalibur (BD Biosciences). To assess the selectin-IgM binding, cells were incubated with a COS-7 supernatant containing E-selectin-IgM, P-selectin-IgM, or control human IgM, washed, and then incubated with biotinylated anti-human IgM (American Qualex). The cells were then washed and stained with either streptavidin-PE (BD Biosciences) alone or streptavidin-allophycocyanin (BD Biosciences) together with 1B11-PE and S7-FITC.

Precipitation with E-selectin-IgG

PSGL-1−/− or wild-type Th1 cells or cultured human T cells were washed three times with PBS and surface-biotinylated in PBS containing 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) (2.5 × 107 cells/ml) at room temperature for 30 min. The cells were then washed three times with PBS and lysed at a density of 3 × 107 cells/ml in cold lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM CaCl2, and 1 mM PMSF) for 30 min. Insoluble materials were pelleted at 15,000 × g for 20 min. The supernatant was aliquoted, and a fraction corresponding to 1 × 107 cells was precipitated with 40 μl of packed protein A-Sepharose. After removal of the Sepharose beads, the lysate was incubated in the presence of 1 mM CaCl2 with 20 μl of protein A-Sepharose preloaded for 4 h at 4°C with 50 μM anti-selectin-IgG or the human IgG. After a 4-h incubation, the beads were washed five times with wash buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM CaCl2). Proteins bound to E-selectin-IgG were eluted with elution buffer (5 mM EDTA, 50 mM Tris (pH 7.4), and 150 mM NaCl) and analyzed by SDS-PAGE and Western blotting.
0.05% Triton X-100). Eluted materials were separated by SDS-PAGE under nonreducing conditions and transferred to an Immobilon-P membrane (Millipore). Membranes were blotted with HRP-conjugated streptavidin (Zymed Laboratories). The membranes were also blotted with a polyclonal anti-mouse PSGL-1 Ab (28), a polyclonal anti-mouse ESL-1 provided by Dr. B. Furie, an anti-mouse CD43 mAb 1B11 (BD Biosciences), an anti-mouse CD44 mAb KM201 (29), an anti-human PSGL-1 mAb KPL-1 (BD Biosciences), or an anti-human CD43 mAb L60 (BD Biosciences), followed by the appropriate HRP-conjugated secondary Abs (all from American Qualex).

To perform Western blot analysis of whole-cell extract, 1 × 10⁶ freshly isolated CD⁴⁺ T or Th1 cells were lysed in lysis buffer. The lysate was resolved by SDS-PAGE under nonreducing and reducing conditions and transferred to an Immobilon-P membrane. For Western blot analysis of mouse CD43-IgG chimeric proteins, 50 ng of protein was separated by SDS-PAGE. Membranes were probed with an anti-CD43 mAb, S7 or 1B11, followed by HRP-conjugated anti-rat IgG. To immunoprecipitate and immunodeplete CD43, eluted materials or total cell lysates were incubated for 2 h with 10 μl of protein G-Sepharose preloaded with 5 μg of 1B11, L60, or control rat or mouse IgG. The incubation with Ab-loaded beads was repeated. The supernatant and beads were analyzed by Western blotting.

**Enzyme treatment**

To remove sialic acid, E-selectin-IgG precipitates or immunoprecipitates were incubated with 1 U/ml neuraminidase from *Clostridium perfringens* (Sigma-Aldrich) in 50 mM sodium phosphate (pH 5.0) at 37°C for 1 h. To remove N-glycan chains, samples were treated with 250 U/ml peptide-N-glycosidase F (PNGase F; Calbiochem) in 50 mM sodium phosphate (pH 7.5) at 37°C overnight. Samples were also treated with 120 μg/ml O-sialoglycoprotein endopeptidase (OSGE; Cederlane Laboratories) at 4°C overnight, and the plates were blocked with 1% BSA in PBS at room temperature. The capillaries containing either CaCl₂ or EDTA and infused into the capillaries at a shear stress of 1 dyn/cm² were infused for 6 min. The shear stress was then increased every 20 s to 128 dyn/cm². The number of cells remaining bound was determined.

**Cell adhesion assays**

The cell adhesion assays were performed as described previously (6). In brief, CD43-IgG, PSGL-1-IgG, or control human IgG (Sigma-Aldrich) (10 μg/ml) was immobilized on 96-well plates (Sigma-Aldrich) at 4°C overnight. The plates were blocked with 1% BSA in PBS at 37°C for 2 h. CHO cells expressing mouse E-selectin (CHO-E cells), provided by Dr. B. Furie, or parental CHO cells resuspended in HBSS containing 2 mM CaCl₂, or 5 mM EDTA were added to the plate and incubated for 20 min at 4°C with rotation (100 rpm). In some experiments, the cells were preincubated with an anti-E-selectin mAb 9A9, provided by Dr. B. Furie, or parental CHO cells resuspended in HBSS containing 2 mM CaCl₂ or 5 mM EDTA, the number of bound cells was determined by photographing the cells and counting them.

**Cell adhesion assays under flow conditions**

Cell adhesion assays under flow conditions were performed according to the method of Nandi et al. (31) with slight modifications. E-selectin-IgG, P-selectin-IgG, CD43-IgG, PSGL-1-IgG, or control human IgG (1 μg/ml) was immobilized on the inside walls of glass capillaries (inner diameter, 0.69 mm; Drummond Scientific) at 4°C overnight. The capillaries were then blocked with 3% BSA for 1 h at room temperature. The capillaries were mounted on the stage of an inverted microscope (Diaphot 300; Nikon) with a ×4 objective. At this magnification, all cells rolling at a fixed position could be monitored. Mouse Th1 cells, CHO-E cells, or parental CHO cells were resuspended at 1 × 10⁶ cells/ml in HBSS containing either CaCl₂ or EDTA and infused into the capillaries at a shear force of 1 dyn/cm². The rate of flow was controlled by a PHD 2000 syringe pump (Harvard Apparatus). In some experiments, the CHO-E cells were preincubated with the anti-E-selectin mAb 9A9 or control rat IgG. Three minutes after the start of infusion, cell images were recorded with a cell-viewing system (SRM-100; Nikon) and video recorder (JVC BR-S600; Victor), and the number of rolling cells passing through a fixed plane (at three-quarters of the capillary tube from the entrance) perpendicular to the capillary axis was counted. The results are expressed as the number of rolling cells per minute. The cells that rolled stably along the wall of the glass capillary tube for at least 3 s were considered to be rolling cells in this assay. For the detachment assays, cells were infused at 1 dyn/cm² for 6 min and the shear stress was increased stepwise every 20 s until it reached 128 dyn/cm². At the end of each shear stress treatment, the number of cells that remained bound was determined.

**Results**

**PSGL-1-deficient Th1 cells roll on E-selectin under flow conditions**

We showed previously that Th1 cells from wild-type mice bind to both P-selectin-IgG and E-selectin-IgG chimeric proteins immobilized on 96-well microtiter plates, whereas PSGL-1−/− Th1 cells do not bind to P-selectin-IgG but bind measurably to E-selectin-IgG (6). Consistent with this result, flow cytometric assays using selectin-IgM chimeric proteins showed that PSGL-1−/− Th1 cells did not bind P-selectin-IgM but bound E-selectin-IgM, albeit at a slightly lower level than wild-type cells (Fig. 1A). We also confirmed that unstimulated CD4⁺ T cells from both wild-type and PSGL-1−/− mice fail to bind P-selectin-IgM and E-selectin-IgM (Fig. 1A). To investigate whether Th1 cells would interact with E-selectin in the absence of PSGL-1 in a more physiological setting, we tested Th1 cells for their ability to roll on E-selectin under flow conditions. When wild-type Th1 cells were infused into capillary tubes coated with selectin-IgG chimeras at 1 dyn/cm², they rolled on E-selectin-IgG as well as on P-selectin-IgG (Fig. 1B).

The addition of EDTA completely abolished the rolling, confirming that this was a calcium-dependent interaction. PSGL-1−/− Th1 cells did not roll on P-selectin-IgG. In contrast, they rolled on E-selectin-IgG, although the number of rolling cells was reduced by ~60%, compared with wild-type cells (Fig. 1B). PSGL-1−/− Th1 cells rolled only on E-selectin-IgG and not on P-selectin-IgG, confirming that PSGL-1 is necessary and sufficient for E-selectin binding under flow conditions.

**FIGURE 1.** PSGL-1-deficient Th1 cells bind and roll on E-selectin under flow conditions. A, P- and E-selectin-IgM binding of unstimulated CD4⁺ T and Th1 cells from wild-type and PSGL-1−/− mice. Cells were incubated with P- and E-selectin-IgM (open histogram) or control human IgM (shaded histogram). Bound selectin IgM was detected using biotinylated anti-human IgM and streptavidin-PE. B, Rolling of wild-type and PSGL-1−/− Th1 cells on P- and E-selectin-IgG. Cells were infused into capillaries coated with P-selectin-IgG, E-selectin-IgG, or human IgG in the presence of calcium or EDTA at a shear stress of 1 dyn/cm². The number of rolling cells was determined. C, Resistance of wild-type and PSGL-1−/− Th1 cells rolling on E-selectin-IgG to detachment by shear stress. Cells were infused into E-selectin-IgG-coated capillaries and allowed to accumulate at a shear stress of 1 dyn/cm² for 6 min. The shear stress was then increased every 20 s to 128 dyn/cm². The number of cells remaining bound at the end of each interval was determined and expressed as a percentage of the cells accumulated before applying increasing shear stress. One of three similar independent experiments is shown.
Th1 cells rolling on E-selectin-IgG exhibited significant resistance to detachment by shear stress, albeit reduced, compared with wild-type cells, with 60% remaining bound at 32 dyn/cm² (Fig. 1C). In this experiment, all cells remaining bound rolled until they finally detached. These data confirm that, although PSGL-1 functions as an E-selectin ligand, there are ligands other than PSGL-1 that can mediate Th1 cell interactions with E-selectin under physiological shear flow.

Several proteins are precipitated with E-selectin-IgG from Th1 cells

To identify E-selectin ligands on Th1 cells, we used E-selectin-IgG bound to protein A-Sepharose as an affinity matrix. Wild-type and PSGL-1−/− Th1 cells were surface-biotinylated, and detergent extracts of these cells were incubated with E-selectin-IgG bound to protein A-Sepharose. The proteins that bound to E-selectin-IgG were eluted with EDTA and subjected to Western blotting with HRP-conjugated streptavidin. Two major bands, which migrated around 130 and 270 kDa under nonreducing conditions, were precipitated from wild-type cells with E-selectin-IgG in the presence of calcium (Fig. 2A). A minor band migrating around 150 kDa was also inconsistently detected. No bands were detected when detergent extracts were incubated with control human IgG in the presence of calcium or with E-selectin-IgG in the presence of EDTA, confirming the specificity of the binding. When the precipitation experiments were performed using PSGL-1−/−/− cells, two bands around 130 and 150 kDa, but not a 270-kDa band, were detected. These results suggested that the 270-kDa band precipitated with E-selectin-IgG from wild-type cells represented PSGL-1. Indeed, a 270-kDa band was detected by Western blotting the E-selectin-IgG precipitate from wild-type cells using anti-PSGL-1 Abs (Fig. 2B).

Besides PSGL-1, ESL-1 has been identified as an E-selectin ligand on mouse neutrophils (8). Whether mouse T cells express ESL-1, as detected by S7 epitope (Fig. 4A), or anti-ESL-1 Ab (Fig. 4C), was examined by flow cytometry. Unstimulated CD4+ T cells and Th1 cells by flow cytometry. Unstimulated CD4+ T cells and Th1 cells expressed ESL-1, whereas Th1 cells expressed both (Fig. 4B). Western blot analysis also showed the expression of the 115-kDa glycoform recognized by S7 in both unstimulated CD4+ T cells and Th1 cells by flow cytometry. Unstimulated CD4+ T cells and Th1 cells expressed ESL-1. Western blot analysis also showed the expression of the 115-kDa glycoform recognized by the mAb 1B11, is expressed preferentially on activated T cells (25). We examined the expression of the S7 and 1B11 epitopes on unstimulated CD4+ T cells and Th1 cells by flow cytometry. Unstimulated CD4+ T cells and Th1 cells expressed ESL-1, whereas Th1 cells expressed both (Fig. 4A).

The 130-kDa protein is cleaved by OSGE and requires sialic acid to bind E-selectin

Most selectin ligands identified to date are sialomucins and require O-linked carbohydrates modified with sLeX-like structures for selectin binding. Some selectin ligands such as ESL-1 require sLeX-linked carbohydrates for selectin binding. To examine whether the 130-kDa protein precipitated with E-selectin-IgG is a member of the sialomucin family, we treated the E-selectin-IgG precipitate from PSGL-1−/− Th1 cells with OSGE. The 130-kDa band was no longer detected after OSGE treatment (Fig. 3), suggesting that the 130-kDa protein is an OSGE-cleavable protein and most likely a sialomucin. The presence of sialic acid was also shown by the shift in the apparent molecular mass after sialidase treatment (Fig. 3). Treatment with PNGase F showed a small shift of the 130-kDa protein (Fig. 3), suggesting that this protein is also N-glycosylated. Only a small amount of the 130-kDa protein, which migrated near 150 kDa after the sialidase treatment, could be reprecipitated with E-selectin-IgG (Fig. 3). In contrast, this protein was reprecipitated after PNGase F treatment, suggesting that N-glycans are not required for E-selectin binding. Together, these results suggest that the 130-kDa protein is likely to be a sialomucin and to require sialic acid for E-selectin binding.

The 130-kDa glycoform of CD43 is expressed on Th1 cells

CD43 is a major sialomucin expressed on T cells. Two major forms of mouse CD43, a 115-kDa and a 130-kDa glycoform, have been identified (25). The 115-kDa glycoform, which is recognized by the anti-CD43 mAb S7, is expressed on all T cells, whereas the 130-kDa glycoform, which is recognized by the mAb 1B11, is expressed preferentially on activated T cells (25). We examined the expression of the S7 and 1B11 epitopes on unstimulated CD4+ T cells and Th1 cells, while the 130-kDa glycoform detected by 1B11 was expressed only in Th1 cells (Fig. 4B). The 130-kDa glycoform of CD43 was immunoprecipitated from Th1 cells with 1B11 (Fig. 4C). Treatment of the immunoprecipitates with OSGE resulted in a complete loss of the band (Fig. 4C), confirming that it is an OSGE-sensitive sialomucin. Sialidase treatment of the immunoprecipitate resulted in a decrease in electrophoretic mobility, thus confirming the presence of sialic acid.

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FIGURE 2. Several proteins were precipitated with E-selectin-IgG from mouse Th1 cells. Wild-type and PSGL-1−/− Th1 cells were surface-biotinylated and their detergent extracts were incubated with E-selectin-IgG (E-IgG) or control human IgG (hIgG) bound to protein A-Sepharose in the presence of calcium or EDTA. Bound proteins were eluted with EDTA, separated by SDS-PAGE under nonreducing conditions, and subjected to Western blotting with HRP-conjugated streptavidin (SA-HRP) (A), anti-PSGL-1 Ab (B), or anti-ESL-1 Ab (C).
Treatment with PNGase F also resulted in a shift, indicating the presence of N-linked carbohydrates. This pattern of shift in electrophoretic mobility after enzyme treatment resembled that observed with the 130-kDa protein precipitated with E-selectin-IgG, raising the possibility that the 130-kDa component of the E-selectin-IgG precipitate may represent CD43.

The 130-kDa component of the E-selectin-IgG precipitate represents CD43

To examine whether CD43 was represented by the 130-kDa component of the E-selectin-IgG precipitate, we subjected the E-selectin-IgG precipitate from PSGL-1-/- Th1 cells to Western blotting with the anti-CD43 mAb 1B11. The 1B11 mAb specifically recognized the 130-kDa band in the E-selectin-IgG precipitate, suggesting that the 130-kDa band contained CD43 (Fig. 5A). The identity of the 130-kDa component was further confirmed by immunoprecipitation and immunodepletion assays with the 1B11 mAb. When the E-selectin-IgG precipitate from PSGL-1-/- Th1 cells was immunoprecipitated with 1B11, the 130-kDa band was mostly depleted from the unbound supernatant, but it was present in the immunoprecipitate (Fig. 5B). In contrast, the 130-kDa band was not detected when control rat IgG was used for the immunoprecipitation; it remained in the supernatant (Fig. 5B). The specific depletion of the 130-kDa band by 1B11 strongly indicates that CD43 made up most or all of the 130-kDa band.

The 130-kDa glycoform of CD43 that the mAb 1B11 recognizes carries core 2-branched O-glycans (25). Because most selectin ligands carry core 2 O-glycans containing the sLeX moiety, we performed flow cytometric analyses to determine whether the expression of the 1B11 epitope was correlated with that of E-selectin ligands. Most Th1 cells expressed the 1B11 epitope, and the cells with high E-selectin-binding activities were enriched in the 1B11high population, compared with the 1B11low population, but no correlation was observed between the expression of the S7 ligands and the E-selectin-binding activities.
epitope and E-selectin-binding activity (Fig. 5C). However, not all
1B11<sup>high</sup> cells had E-selectin-binding activity, suggesting that only
part of the 130-kDa glycoform of CD43 is a selectin-binding form.

**CD43 is an E-selectin ligand on human T lymphoblasts**

To investigate whether human T cell CD43 would function as an
E-selectin ligand, we prepared T lymphoblasts from PBMCs of
healthy donors by stimulating them with anti-CD3 and culturing
them in the presence of IL-2 in X-VIVO 15 medium. As shown
previously (32), these cells had high E-selectin-binding activity
(data not shown). The cells were then surface-biotinylated, and
their detergent extracts were precipitated with E-selectin-IgG.
Three major bands, migrating at around 130, 180, and 270 kDa
under nonreducing conditions, were detected (Fig. 6A). A 270-kDa
band was detected by Western blotting the E-selectin-IgG precip-
itate with anti-PSGL-1 Abs (Fig. 6B), suggesting that PSGL-1 was
at least one component of the 270-kDa band of the E-selectin-IgG
precipitate. To determine whether CD43 contributed to the 130-
kDa component of the E-selectin-IgG precipitate from cultured
human T cells, we subjected the E-selectin-IgG precipitate to
Western blotting with the anti-human CD43 mAb L60, which spe-
cifically recognized the 130-kDa component (Fig. 6C). In addition,
L60 but not control mouse IgG, largely depleted the 130-kDa band
from the E-selectin-IgG precipitate (Fig. 6D), indicating that on
cultured human T cells too, CD43 is an E-selectin ligand.

**CD43 mediates E-selectin-dependent cell rolling under flow
conditions**

We next investigated whether CD43 could mediate E-selectin-de-
pendent cell adhesion. To this end, we first prepared mouse and
human CD43-IgG chimeric proteins in CHO-derived CD7II cells
stably expressing both FucT-VII and C2GnT (27), which together
generate core 2 O-glycans with sLe<sup>x</sup>-like structures on the chi-
meric proteins. The mouse and human PSGL-1-IgG chimeric pro-
teins were generated similarly. The proteins were also made in
CHO cells that did not express FucT-VII or C2GnT, and used as
controls. The mouse CD43-IgG generated in CD7II cells expressed
the 1B11 epitope but not the S7 epitope, whereas the control
CD43-IgG expressed the S7 epitope but not the 1B11 epitope (Fig.
7A). As shown in Fig. 7B, although the parental CHO cells did not
bind to any of the chimeric proteins, the CHO-E cells bound to the
PSGL-1-IgG and CD43-IgG of both mouse and human origin gen-
erated in the CD7II cells, but not to the control PSGL-1-IgG and
CD43-IgG. The binding was almost completely abrogated by the
addition of EDTA (data not shown), confirming that it was cal-
cium-dependent. The binding of CHO-E cells to the CD7II-
generated CD43-IgG and PSGL-1-IgG was also inhibited by
pretreating the cells with the anti-E-selectin mAb 9A9, but not
with control rat IgG, verifying that the binding was dependent
on E-selectin (Fig. 7C).

We next investigated whether CD43 could mediate E-selectin-de-
pendent cell rolling under flow conditions. When CHO-E cells
were infused into capillary tubes coated with the chimeric proteins
under a shear stress of 1 dyn/cm<sup>2</sup>, the CHO-E cells rolled on the
CD43-IgG and the PSGL-1-IgG generated in CD7II cells, but not
on the control chimeric proteins (Fig. 7D). The rolling of CHO-E
cells was completely inhibited by pretreating the cells with the
anti-E-selectin blocking mAb 9A9 but not with control rat IgG,
verifying that the rolling was dependent on E-selectin (Fig. 7E).
Taken together, these data suggest that CD43, when appropriately
modified by a set of glycosyltransferases, functions as an E-selec-
tin ligand under flow conditions.

**Discussion**

In this study, we identified a 130-kDa glycoprotein ligand for E-
selectin on mouse Th1 cells using an E-selectin-IgG chimera as an
affinity probe, and identified this glycoprotein as the 130-kDa gly-
coform of CD43. Human CD43 also represented the 130-kDa gly-
coprotein precipitated with an E-selectin-IgG chimera from activ-
ated human T cells. Furthermore, using CD43-IgG chimeric pro-
teins generated in CHO cells expressing FucT-VII and C2GnT,
we showed that CD43 could support E-selectin-dependent cell
rolling under flow, indicating that CD43 can serve as an E-selectin
ligand on activated T cells and potentially mediate the migration of
activated T cells to sites of inflammation.

The identity and physiological contributions of E-selectin li-
gands are not well established. We showed previously that the
PSGL-1 expressed on mouse Th1 cells functions as an E-selectin
ligand (6). Our study shows that PSGL-1 is indeed precipitated
from mouse Th1 cells with an E-selectin-IgG chimera, confirming
biochemically that PSGL-1 is an E-selectin ligand. We have also
reported that ESL-1, which was identified as an E-selectin ligand
on mouse neutrophils (8), is also precipitated with E-selectin-IgG
from mouse Th1 cells, suggesting that ESL-1 can serve as an E-
selectin ligand on T cells as well. In addition to these two ligands,
we report that a 130-kDa glycoprotein is another E-selectin
ligand under flow conditions.
similar size. This difference is likely to be due to variations in the culture conditions: in the other study, lymphocytes were activated by two cycles of anti-CD3 stimulation, but in our study, the cells were stimulated only once. In addition, CD44, a hyaluronan-binding cell surface molecule, has recently been shown to function as an E-selectin ligand on mouse neutrophils (13). Although mouse Th1 cells express high levels of CD44, we did not detect any bands in Western blots of the E-selectin-IgG precipitate probed with the anti-CD44 mAb KM201 (data not shown), suggesting that CD44 may not act as an E-selectin ligand on mouse Th1 cells.

The function of CD43 remains elusive (33). Because of its extended structure and strong negative charge, CD43 is thought to function as an antiadhesive ligand and provide a repulsive barrier around a cell. Targeted disruption of CD43 in cell lines and mice leads to increased cell adhesion and proliferation (21, 34) as well as increased T cell migration to secondary lymphoid organs in vivo (22). In contrast, the expression of CD43 on T cells enhances their adhesion to APCs, suggesting a proadhesive role for CD43 (35). Indeed, several potential ligands for CD43 have been reported, including ICAM-1 (36), galectin-1 (37), and sialoadhesin (38). In this study, we showed that CD43 on activated T cells can serve as a proadhesive ligand for E-selectin. Tumor-derived CD43 has also been shown to bind E-selectin (14, 39, 40). It needs to be clarified whether any of these potential ligands function in vivo.

Interestingly, both antiadhesive and proadhesive functions have been described for several sialomucins, such as CD34, podocalectin, and endomucin, which can serve as L-selectin ligands when appropriately modified (41–43).

The ability of CD43 to act as an E-selectin ligand is regulated by cell-specific posttranslational modifications. E-selectin recognizes sialylated and fucosylated carbohydrate structures represented by sLeX. The biosynthesis of sLeX requires the sequential action of several glycosyltransferases including /H9251-1,3-fucosyltransferases. FucT-VII plays a dominant role in selectin ligand generation in T cells (44). Although FucT-VII is not expressed in naive CD4<sup>+</sup>/H11001 T cells, its mRNA is augmented when the cells are activated in the presence of the Th1-polarizing cytokine IL-12 (45). In addition, the expression level of C2GnT, which generates core 2 structures, increases upon T cell activation, regardless of the presence of polarizing cytokines (45). The expression of the 130-kDa glycoform of CD43 detected by 1B11 depends on the activity of C2GnT, but not FucT-VII, which explains why not all 1B11<sub>high</sub> cells have E-selectin-binding activity. Our data suggest that in mouse Th1 cells and cultured human T cells that express both FucT-VII and C2GnT, CD43 is one of the core proteins modified by these enzymes to carry selectin-binding fucosylated core 2 O-glycans. Thus, although the 130-kDa glycoform of CD43 that carries core

**FIGURE 7.** CD43 mediates E-selectin-dependent cell rolling under flow conditions. A. Expression of S7 and 1B11 epitopes on mouse CD43-IgG generated either in CHO-derived CD7II cells that express both FucT-VII and C2GnT or control CHO cells. Mouse CD43-IgG generated in CD7II or control CHO cells was separated by SDS-PAGE and blotted using S7 and 1B11. B. Adhesion of CHO-E cells to CD43- and PSGL-1-IgG chimeras. CHO-E or control CHO cells were added to 96-well plates coated with mouse or human CD43- or PSGL-1-IgG, generated either in CD7II or control CHO cells. The plates were rotated for 20 min, the unbound cells were removed, and the number of bound cells was determined. Values are means ± SD from triplicate wells. C. Effect of the anti-E-selectin mAb 9A9 on CHO-E cell adhesion to CD7II-generated CD43- or PSGL-1-IgG chimeras. CHO-E or control CHO cells were incubated with or without the anti-E-selectin mAb 9A9 or control rat IgG for 30 min and then added to the wells. Values are means ± SD from triplicate wells. D. Rolling of CHO-E cells on CD43- and PSGL-1-IgG chimeras. CHO-E and control CHO cells were infused into capillaries coated with mouse or human CD43- or PSGL-1-IgG that had been generated either in CD7II or control CHO cells, at a shear stress of 1 dyn/cm<sup>2</sup>. The number of rolling cells was determined. E. Effect of the anti-E-selectin mAb 9A9 on CHO-E cell rolling on mouse or human CD43- or PSGL-1-IgG chimeras generated in CD7II cells. CHO-E cells were incubated with or without the anti-E-selectin mAb 9A9 or control rat IgG for 30 min and then infused into the capillaries.
2 O-glycans has been implicated in an adhesive process (46), fucosylation of this glycoform may switch the molecule to a proadhesive selectin ligand.

In vitro adhesion assays have shown that CD43, when appropriately modified by FucT-VII and C2GnT, can support cell tethering and rolling under flow conditions. The molecules that support tethering and rolling, such as L-selectin, PSGL-1, and α4 integrins, are typically concentrated at the tip of folds or processes, called microvilli, on the cell surface. CD43 is also localized on microvilli (47), favoring the view that CD43 is an adhesion molecule mediating tethering and rolling interactions. ESL-1 is also located on microvilli, albeit mostly on their sides (48), whereas CD44 is located on the planar cell surface (49). It is interesting to speculate that the difference in the localization of each E-selectin ligand may confer on them distinct roles in E-selectin-mediated adhesive processes.

In conclusion, our study provides evidence for the role of CD43 as an E-selectin ligand on activated T cells. The identification of CD43 as one of the E-selectin ligands, in addition to previously identified ligands, such as PSGL-1 and ESL-1, will allow the clarification of the distinct and redundant roles of these ligands in various inflammatory conditions in vivo.

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Disclosures
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References
1. Kansas, G. S. 1996. Selectins and their ligands: current concepts and controver-
selectin, thymus- and activation-regulated chemokine/CCL17, and intercel-
lar adhesion molecule-1 are constitutively coexpressed in dermal microves-
gand-1-deficient mice have impaired leukocyte tethering to E-selectin under flow.


