Immunity, Vol. 8, 373–381, March, 1998, Copyright ©1998 by Cell Press

Negative Regulation of T Cell Homing by CD43

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Summary

We report that the cell surface mucin CD43 acts as an anti-adhesin on T lymphocytes. CD43-deficient murine lymphocytes homed significantly more frequently to secondary lymphoid organs than wild-type cells. Intravital microscopy of peripheral lymph node venules revealed that CD43-deficient lymphocytes were twice as likely to tether, roll, and stick than wild-type cells. This effect was due to CD43 interference with the homing receptor, L-selectin, and was most pronounced in venules with low L-selectin ligand density. In vitro, CD43-deficient cells tethered to L-selectin ligands more efficiently and rolled more slowly than wild-type lymphocytes. Thus, CD43 exerts a negative regulatory effect on T cell trafficking by counterbalancing L-selectin–mediated adhesion.

Introduction

CD43 is a large, negatively charged sialoglycoprotein expressed by virtually all leukocytes, except mature B cells (reviewed by Remold-O'Donnell and Rosen, 1990). Previous studies have shown that CD43 expression on the cell surface attenuates both cell-to-cell and cell-to-substrate binding under static conditions. CD43, when transfected into HeLa cells, inhibits their adhesion to T cells (Ardman et al., 1992), and a CD43-deficient human T cell line shows increased homotypic adhesion in culture and enhanced binding to fibronectin and human immunodeficiency virus 1 gp120 (Manjunath et al., 1993). Furthermore, splenocytes from CD43-deficient mice bind better to both fibronectin and intercellular adhesion molecule 1 (ICAM-1) than those from wild-type littermates (Manjunath et al., 1995). These results suggest that CD43 diminishes T lymphocyte adhesion irrespective of the specific adhesion pathway responsible (e.g., CD4, β1, or β2 integrins), and that CD43 provides a general anti-adhesive barrier on cell surfaces, impeding the function of several types of receptors. Indeed, the extracellular portion of CD43, which extends 45 nm from the cell surface and displays a negatively charged O-linked sialic acid residue on every third amino acid (reviewed by Fukuda, 1991), seems especially suited to prevent surface receptors from reaching their ligands.

However, it has not been tested whether CD43 exerts similar effects under the conditions of hydrodynamic shear that are encountered in the blood stream. Moreover, results from several previous studies are also consistent with a pro-adhesive function for CD43, for instance by means of an intracellular signal responsible for increasing the affinity or avidity of LFA-1 for ICAM-1 (DeSmet et al., 1993; Sanchez-Mateos et al., 1995; Sperling et al., 1995). A recent report has also shown that a monoclonal antibody (mAb) to CD43 can inhibit lymphocyte homing to secondary lymphoid organs (McEvoy et al., 1997), a finding that is equally consistent with antibody blockade of a pro-adhesive function of CD43 or with antibody-induced signaling effects. Thus, the question whether the mere presence of CD43 helps or hinders lymphocyte recruitment into tissues has yet to be specifically addressed.

Lymphocyte trafficking from the blood stream into and through lymphoid organs is an adhesion receptor-regulated process that is vital for normal immune surveillance in mammals (reviewed by Springer, 1994; Butcher and Picker, 1996). Recent intravital microscopy studies of normal murine lymphocytes in peripheral lymph nodes (PLN) and Peyer's patches (PP) have shown that lymphocyte homing involves organ-specific multistep cascades of adhesion and signaling events in specialized blood vessels termed high endothelial venules (HEV) (Bargatze et al., 1995; von Andrian, 1996; Wagner et al., 1996; Warnock et al., 1998). In noninflamed HEV of both lymphoid organs, initial tethering and rolling of circulating cells are mediated by lymphocyte L-selectin (CD62L), which binds to the peripheral node addressin (PNAd), a complex carbohydrate determinant that is selectively recognized by the mAb MECA-79 (Streeter et al., 1988; Berg et al., 1991, 1993). A fraction of these rolling cells are subsequently triggered to "stick" via propagation of a G-protein–linked signal that results in functional up-regulation of the β2 integrin LFA-1 (Warnock et al., 1998). Studies to date have focused on the pro-adhesive ligands necessary for successful completion of the sequential adhesive steps that promote lymphocyte homing. An anti-adhesive barrier functioning at any or all of these steps could be expected to affect the ultimate extravasation of cells from the blood into lymphoid tissue.

To elucidate the role of CD43 in physiologic lymphocyte trafficking, we compared the homing efficiency of lymphocytes harvested from wild-type and CD43-deficient mice. To measure directly the effect of CD43 expression on completion of the multistep adhesion cascade, we used intravital fluorescence microscopy of murine PLN microvessels (von Andrian, 1996) and in vitro studies of lymphocyte adhesion to purified PNAd under flow (Diacovo et al. 1996). Our findings show that CD43 negatively regulates T lymphocyte homing to secondary lymphoid organs. In PLN, CD43 exerts this effect

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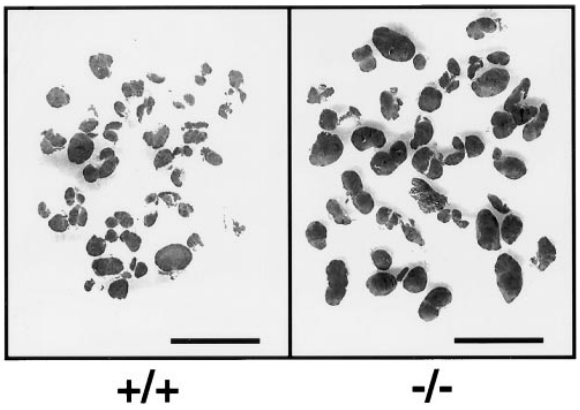

Figure 1. Increased Size of PLN in CD43 Mutant Mice

Typical micrographs of hematoxilin–eosin–stained PLN sections through the central plane (i.e., the largest cross-sectional area in each series). The cross-sectional area (mean ± SEM) of wild-type PLN measured 0.85 ± 0.09 mm^2 (left), whereas PLN from $CD43^{-/-}$ mice measured 1.61 ± 0.13 mm^2 (right) ($P < 0.05$, Student's t test). Scale bars, 5 mm.

specifically through interference with L-selectin–mediated tethering and rolling.

Results and Discussion

PLN in CD43-Deficient Mice Are Larger Than Those in Wild-Type Mice

Macroscopically, PLN from $CD43^{-/-}$ donors housed in specific pathogen–free/virus antibody–free conditions appeared larger than those from wild-type mice. The cross-sectional areas of PLN from $CD43^{-/-}$ animals were, on average, nearly 2-fold greater than those of wild-type PLN (Figure 1). However, PLN showed no discernible differences in anatomic organization or the general appearance of B and T lymphocyte zones (Manjunath et al., 1995). These findings suggest that CD43 may be a constitutive determinant of the ultimate size of PLN. One possible mechanism that could be responsible for this observation is the involvement of CD43 in the regulation of lymphocyte entry from the blood. To test this possibility, we compared the ability of wild-type and mutant lymphocytes to home to secondary lymphoid organs.

CD43 Attenuates T Lymphocyte Homing

AKR/J mice were the designated recipients of lymphocytes isolated from PLN of $CD43^{-/-}$ mice and wild-type littermates. AKR/J mice expressed the Thy1.1 (CD90) T cell marker, whereas T cells from donor animals (C57BL/6 × 129Sv/J1) displayed the Thy1.2 allotype (Ledbetter and Herzenberg, 1979). Thus, it was possible to identify and quantitate CD43-positive and -negative donor T cells in lymphocyte suspensions of recipient organs by two-color flow cytometry. Two hours after injection of equal numbers of lymphocytes from wild-type and $CD43^{-/-}$ donors, approximately 45% more CD43-deficient than wild-type T cells were recovered from the spleen, PLN, and mesenteric lymph nodes

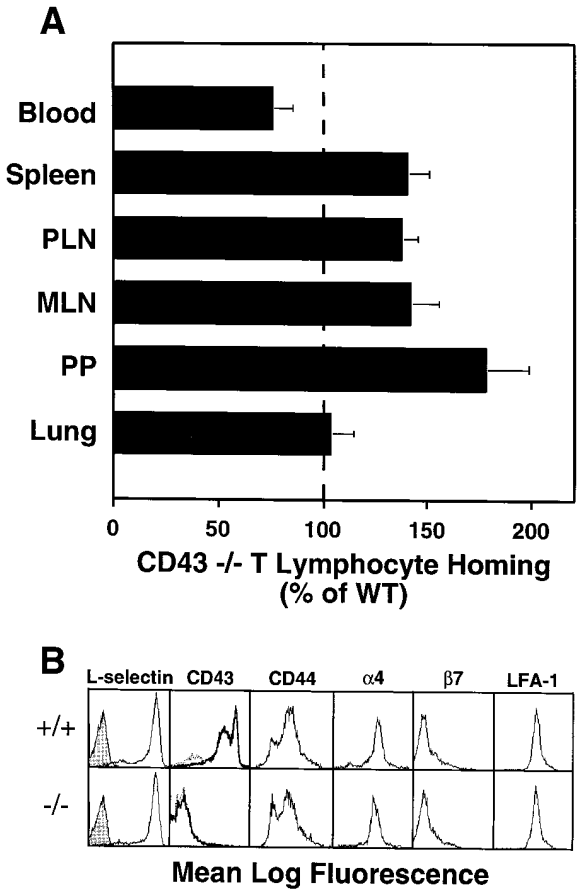

Figure 2. $CD43^{-/-}$ T Cells Home More Efficiently to Secondary Lymphoid Organs but Express Equivalent Levels of Homing Receptors

(A) $CD43^{-/-}$ T cells home more efficiently to secondary lymphoid organs. Data represent the mean ± SEM of T cell homing ratios ([frequency of $CD43^{-}Thy1.2^{+}$]/[frequency of $CD43^{+}Thy1.2^{+}$], corrected for differences in input) from eight independent experiments. Homing ratios in the lung are from three experiments.

(B) Flow cytometric analysis of input lymphocytes from one representative homing experiment. PLN lymphocytes from $CD43^{-/-}$ mice (bottom row) and wild-type littermates (top row) showed similar patterns of expression of L-selectin, CD44, α4, and β7 integrins, and LFA-1 α chain. Background staining with biotinylated rat immunoglobulin G followed by PE-streptavidin (shaded areas at left) was similar to that obtained with PE-goat anti-rat-immunoglobulin G $F(ab)_2$ (data not shown). Histograms of anti-CD43 staining are shown for all PLN lymphocytes (dashed lines) and after gating for Thy1.2-positive T cells (solid lines).

(MLN) of recipients (Figure 2A). Homing of CD43-deficient T cells to PP was 1.8-fold increased compared with that of wild-type cells. Correspondingly fewer CD43-deficient T cells were found in the peripheral blood of recipient mice, and equivalent numbers of CD43-positive and -deficient T cells were recovered from lung tissue. Similar results were obtained when wild-type and mutant lymphocytes were differentially stained with green and red fluorescent dyes (calcein-AM and TRITC, respectively) and then injected into wild-type recipients (data not shown). These results indicate that CD43 deficiency specifically accelerates the redistribution of transfused T cells from the blood to secondary lymphoid

tissues. The observation that T cell accumulation was not enhanced in the pulmonary circulation suggests further that the faster homing of CD43-deficient T cells was not a consequence of a nonspecific increase in the mutant cells' stickiness or of differences in T cell size or deformability that could prolong cell retention in capillaries.

Homing Receptor Expression and L-Selectin Binding to a Soluble Ligand Are Not Affected by CD43 Deficiency

To test whether the enhanced homing of CD43-deficient lymphocytes resulted from increased expression of known homing receptors or from occult cell activation, we compared the expression of CD44, L-selectin, LFA-1, and the α4 and β7 integrins. Each of these molecules was equivalently expressed in both strains (Figure 2B). Because CD43 is abundantly expressed on the surface of T lymphocytes and carries a large portion of the total cell surface carbohydrate (Standring et al., 1978), we also investigated the possibility that CD43 deficiency may have altered L-selectin lectin activity. This hypothesis was tested on wild-type and CD43-deficient lymphocytes by correlating L-selectin expression levels with L-selectin–dependent binding of fluorescein isothiocyanate (FITC)–conjugated polyphosphomannan ester (PPME-FITC), a soluble polysaccharide from *Hansenula hostii* (Yednock et al., 1987). The average number of L-selectin molecules per cell was 85,000 $\pm$ 25,000 (mean $\pm$ SD) on wild-type lymphocytes, and 92,000 $\pm$ 31,000 receptors were found on CD43-deficient cells. L-selectin–specific PPME-FITC binding to CD43-deficient cells did not differ from wild type; the mean fluorescence intensity (using a linear scale setting) of PPME-FITC per 100,000 receptors was 39.7 $\pm$ 11.3 (mean $\pm$ SEM) for wild-type cells versus 34.7 $\pm$ 2.8 for CD43$^{-/-}$ cells. These results exclude the possibility that the enhanced homing to lymphoid organs of CD43-deficient lymphocytes was a consequence of exaggerated L-selectin expression or lectin activity at the cell surface.

CD43 Interferes with Lymphocyte Adhesion to PLN Venules In Vivo

To determine at which step CD43 interferes with the coordinated process of lymphocyte homing, we employed intravital microscopy of PLN microvessels to measure rolling and sticking fractions for wild-type and CD43-deficient cells. Lymphocyte samples from both strains were injected successively into the same recipient, with 90–120 min allowed between injections to permit fluorescent cells to clear from the blood stream. CD43-deficient lymphocytes rolled more frequently than wild-type cells in virtually all venules examined (Figure 3A). In contrast, CD43 did not affect the frequency at which rolling cells arrested; the percentage of rolling wild-type and mutant cells that became stationary for at least 30 s was 6.3% $\pm$ 7.1% (mean $\pm$ SEM) and 7.3% $\pm$ 4.6%, respectively. However, since the rolling fractions of CD43-deficient lymphocytes were much higher than those of wild-type cells, the frequency of sticking in the total (i.e., rolling plus noninteracting) flux

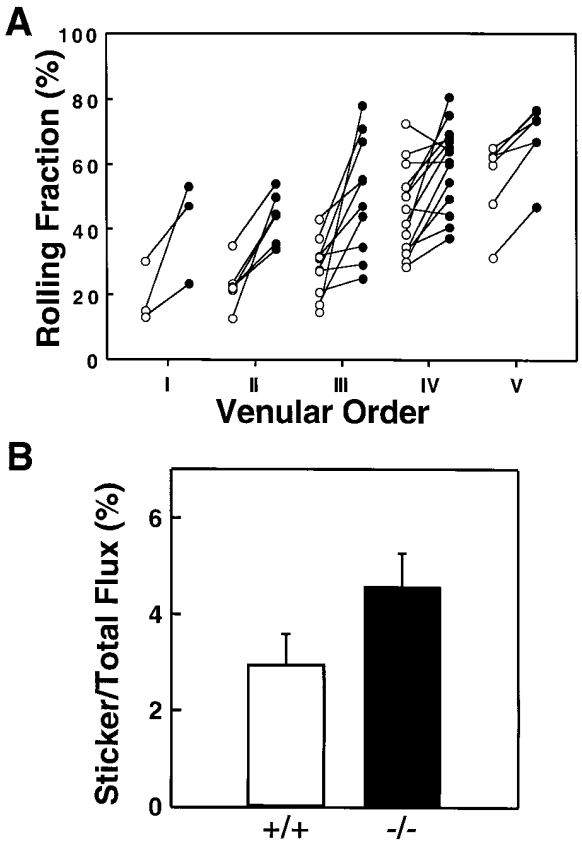

Figure 3. Enhanced Rolling and Sticking of CD43$^{-/-}$ Lymphocytes in PLN Venules

(A) Rolling fractions (the percentage of rolling cells in the total flux of cells passing a venule) of CD43$^{-/-}$ lymphocytes (filled circles) and wild-type cells (open circles) were determined in different venular orders. Each data pair connected by solid lines indicates rolling fractions of both cell subsets in the same venule.

(B) Sticking fractions of wild-type (+/+) and CD43 deficient (−/−) PLN lymphocytes were determined as the percentage of cells that arrested for at least 30 s in the total flux of cells from each subset that passed each venule during the observation period. Venules in which fewer than 10 rolling cells were observed were not included in this analysis. Means $\pm$ SEM of sticking fractions from 26 venules of seven animals are shown. The difference is statistically significant ($P < 0.05$, Student's t test).

of cells that passed through a given venule was significantly enhanced (4.6% $\pm$ 0.7% for CD43$^{-/-}$ vs. 2.9% $\pm$ 0.6% for wild-type; $P < 0.05$) (Figure 3B).

Previous studies with wild-type lymphocytes have shown that rolling in PLN is mediated nearly exclusively by L-selectin, whereas sticking requires the engagement of LFA-1 (Warnock et al., 1998). The observation that the most apparent effect of CD43 deficiency is a dramatic increase in rolling suggests that the acceleration in PLN homing of CD43-deficient cells may have resulted largely from greater efficieny of primary adhesion events rather than enhancement of integrin-mediated arrest. Since CD43-deficient lymphocytes expressed a similar number of L-selectin receptors with equivalent lectin activity (discussed above), we determined whether the enhanced rolling of the CD43-deficient cells could have resulted from their use of an alternate tethering pathway

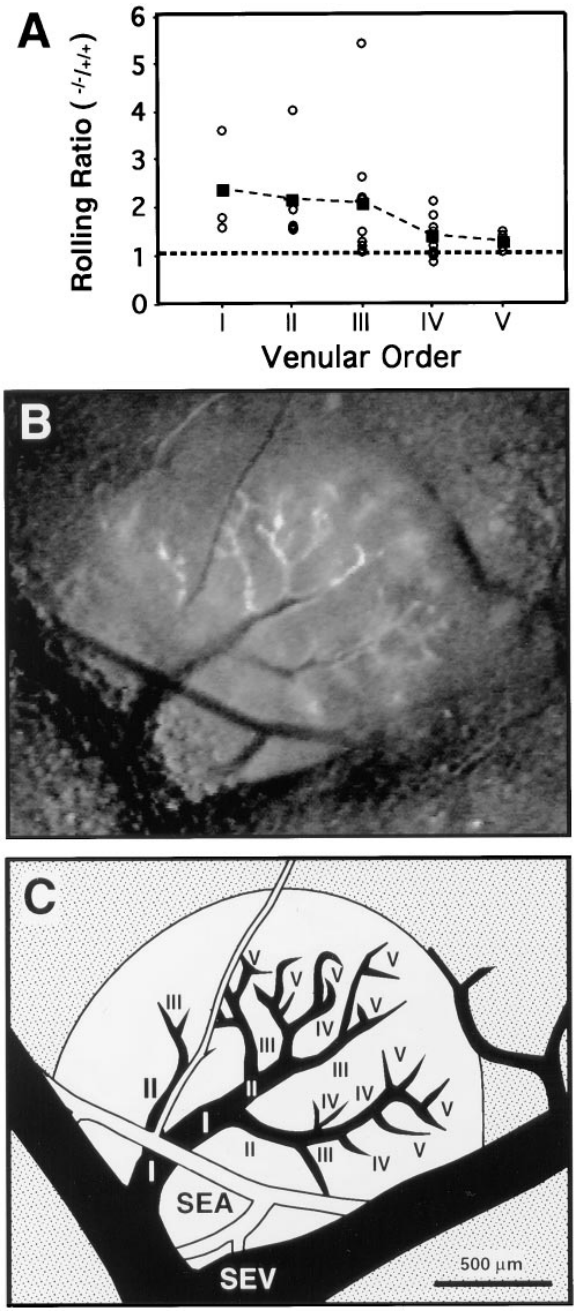

Figure 4. The Enhanced Ability of CD43$^{-/-}$ Lymphocytes to Roll in PLN Venules Is More Pronounced in Venules with Low Expression of L-Selectin Ligands

(A) Rolling fractions of CD43$^{-/-}$ cells in individual venules are presented as the fold increase over wild-type rolling fractions (rolling ratio). The bold dotted line indicates equivalent rolling fractions of wild-type and CD43$^{-/-}$ cells; the dashed line connects average rolling ratios (filled squares) in different venular orders. The raw data used for calculations of rolling ratios are the same as those in Figure 3A.

(B) Fluorescent micrograph illustrating the distribution of the MECA-79 antigen (the main component of PNAd) in the venular tree of a typical subiliac PLN.

(C) A schematic drawing of the micrograph illustrates venular orders (Roman numerals) in the PLN (white area), which is embedded in fatty tissue (dotted area). No staining was detectable in extralymphoid vessels such as the superficial epigastric artery (SEA) and vein (SEV) or their tributaries. Similar results were observed in six independent experiments (Table 1).

independent of L-selectin. Pretreatment of lymphocytes with the L-selectin–blocking mAb Mel-14 prior to injection virtually abrogated rolling of wild-type and CD43-deficient lymphocytes in all PLN venules (mean ± SD of rolling fractions: 2.7% ± 4.8% for CD43$^{-/-}$ vs. 2.5% ± 4.5% for wild-type; $n \geq 3$ experiments each), eliminating the possibility that CD43 deficiency had unmasked other rolling receptors on the cell surface.

The Anti-Adhesive Effect of CD43 Is Inversely Correlated to PNAd Density

It has been observed previously that the frequency of L-selectin–mediated lymphocyte rolling in PLN varies with the venular order—the branching generation of vessels upstream from the draining epigastric vein (von Andrian, 1996; Warnock et al., 1998). This observation was confirmed in the present experiments with both wild-type and mutant lymphocytes (Figure 3A); rolling fractions were higher in postcapillary and small collecting venules in the paracortex (orders III–V) than in the large collecting venules in the medulla (orders I and II). Interestingly, the difference in rolling between wild-type and CD43-deficient lymphocytes was most striking in collecting order I and II venules (Figure 4A). The advantage of CD43$^{-/-}$ cells was much less marked, though consistently detectable, in the small, postcapillary order IV and V HEV.

To determine whether this spatial distinction was related to topographic differences in the expression of PNAd, we injected fluoresceinated mAb MECA-79 into mice and assessed the localization of its antigen in PLN from video recordings of venular trees (Figures 4B and 4C and Table 1). MECA-79 staining was continuous and intense at the luminal surface of most order III and all order IV and V venules. In contrast, most order II venules displayed diminished and/or discontinuous expression of the MECA-79 antigen, and several bound no mAb at all. No MECA-79 accumulation was detected in order I medullary venules, except in one instance in which a single endothelial cell appeared to be stained. There also was no detectable expression of the MECA-79 antigen in arterioles or capillaries. These data suggest that there is an inverse relationship between the anti-adhesive effect of CD43 and the relative abundance of endothelial L-selectin ligands; CD43 confers a rolling disadvantage, especially in those venules in which PNAd density is low. Conversely, the attenuating effect of CD43 is partially overcome in HEV with very high PNAd density.

CD43 Promotes Detachment from HEV and Attenuates L-Selectin Tethering

The increase in L-selectin–mediated rolling of CD43-deficient lymphocytes and the apparent spatial preference for this phenomenon in lower-order venules might be explained by two possible mechanisms: CD43-deficient cells that tether in high-order venules may detach less frequently and thus continue to roll more efficiently into downstream collecting venules, and/or CD43-deficient cells may be more efficient at using L-selectin to establish primary tethers to these larger vessels, where

Table 1. Analysis of MECA-79 Staining in PLN Venules

	Venular Order in PLN				
	I	II	III	IV	V
Number of venules	6	12	33	51	17
Diameter (μm)	85 ± 24	61 ± 17	34 ± 16	27 ± 10	16 ± 4
Intensity of MECA-79 staining					
0	5 (83.33%)	3 (25%)	0	0	0
+	1 (16.67%)	9 (75%)	2 (6.06%)	0	0
++	0	0	31 (93.9%)	51 (100%)	17 (100%)

Fifty micrograms of CFSE-conjugated mAb MECA-79 was injected intravenously, and fluorescent staining of PLN venules was scored in each of six animals.
0, no detectable fluorescence above background; +, detectable, but weak or discontinuous fluorescent label; ++, bright label of the entire luminal surface.

PNAd density is lower. Tracking of individual lymphocytes that had formed initial tethers in postcapillary order IV and V venules revealed that before detaching from the vessel wall, CD43-deficient cells rolled a 1.6-fold greater distance, on average, than wild-type cells (353 ± 78 μm vs. 223 ± 46 μm; $P < 0.01$) (Figure 5A). In addition, CD43-deficient cells formed more primary tethers in low-order (I and II) venules (tethering fraction: 4.7 ± 1.8% for CD43-deficient vs. 2.2 ± 1.5% for wild-type; $P < 0.05$) (Figure 5B). Thus, CD43 expression both enhances the detachment of cells from the higher-order venules and diminishes their ability to tether downstream in the lower-order venules.

CD43 Attenuates Lymphocyte Tethering to L-Selectin Ligands in Vitro

To exclude the possibility that any observed effects on lymphocyte tethering and rolling may have resulted from indirect mechanisms that can occur in vivo, such as variations in fluid shear (Finger et al., 1996) or lymphocyte interactions with erythrocytes (Schmid-Schönbein et al., 1980) or activated platelets (Diacovo et al., 1996), we tested the ability of wild-type and CD43-deficient PLN lymphocytes to interact with immobilized, affinity-purified PNAd in a flow chamber. Tethering of CD43-deficient lymphocytes to PNAd in the absence of other blood components was significantly increased over that of wild-type cells (17.3 ± 2.2 cells/min/mm^2 vs. 10.4 ± 1.8 cells/min/mm^2, respectively; $P < 0.001$) (Figure 6A). In addition, the average rolling velocity of CD43-deficient cells was modestly, but significantly, lower than that of wild-type cells on identical PNAd substrates (28.0 ± 4.4 μm/s vs. 32.9 ± 5.6 μm/s, respectively; $P < 0.05$) (Figure 6B). Both CD43-deficient and wild-type lymphocyte interactions with PNAd were completely inhibited by anti-L-selectin mAb (data not shown). Taken together, our in vivo and in vitro observations strongly suggest that CD43 interferes with lymphocyte trafficking to PLN by decreasing the ability of L-selectin to initiate and maintain adhesive interactions with PNAd.

CD43 Hinders Rolling in PP HEV

Because L-selectin also participates in lymphocyte tethering and rolling on mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) in PP and MLN (Berg et al., 1993; Bargatze et al., 1995; Wagner et al., 1996), our findings would predict that CD43 exerts a similar inhibitory effect in these mucosa-associated lymphoid organs. Indeed, we observed that 56% ± 4.5% (mean ± SEM) of all CD43-deficient lymphocytes rolled in PP (23 HEV in three mice), whereas the rolling fraction of wild-type cells in the same vascular beds was 44% ± 4.6% ($P < 0.05$; data not shown). However, adhesion molecules that are distinct from L-selectin, such as the α4β7 integrin, contribute to lymphocyte adhesion in PP and MLN HEV (Bargatze et al., 1995; Wagner et al., 1996), and other unknown molecular mechanisms may be involved in lymphocyte migration to the spleen. Thus it is likely that the effect of CD43 on short-term homing to these secondary lymphoid tissues was not due solely to interference with L-selectin.

Putative Mechanisms and Possible Consequences of CD43-Mediated Anti-Adhesion

Previous studies have shown that CD43 cross-linking can trigger an intracellular signal in lymphocytes (Mentzer et al., 1987; Axelsson et al., 1988), and in a recent report, antibody binding to CD43 on the surface of normal murine T cells was found to inhibit their homing to PLN and PP (McEvoy et al., 1997). CD43-induced transmission of an intracellular signal that inhibits T lymphocyte recruitment to PLN and PP could explain how CD43 ligation curbs T cell homing, provided that lymphocytes can encounter physiologic CD43 ligand(s). However, results from our experiments argue against the presence of a pro-adhesive ligand for CD43, at least in the intravascular compartment of noninflamed PLN or PP. Moreover, CD43 significantly interfered with a nearly instantaneous event: L-selectin–dependent tethering of freely flowing cells to PNAd is known to occur more rapidly than the temporal resolution of our video system of 0.03 s/frame (Alon et al., 1997). It is questionable whether intracellular signals propagated through CD43 could be sufficiently fast to affect these primary adhesive interactions. Rather, the anti-adhesive effect of CD43 on L-selectin–mediated tethering most likely derives from the large size (~45 nm) and considerable negative charge of its ectodomain (Cyster et al., 1991). In addition, lymphocyte tethering to PNAd under flow requires L-selectin concentration on the tips of microvillous membrane protrusions (von Andrian et al., 1995), where coexpression of CD43 (Yonemura et al., 1993)

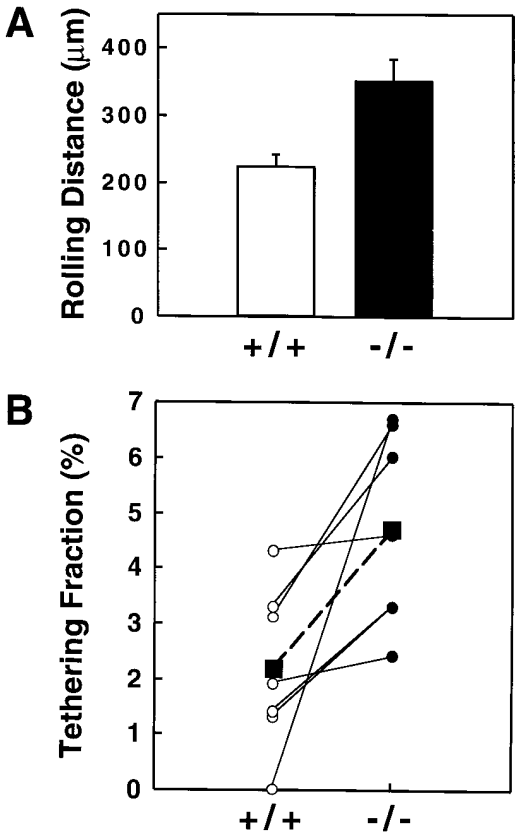

Figure 5. Delayed Detachment and Increased Tethering of CD43-Deficient Lymphocytes In Vivo

(A) Individual lymphocytes that began to roll immediately after entering postcapillary order IV and V venules were traced until they detached from the vascular wall (cells that became stuck were not included in this analysis). The rolling distance (the distance from the point of attachment to the site of release) was measured by computer-assisted video analysis (Pries, 1988). Data shown are means ± SEM.

(B) Increased tethering of mutant cells in medullary collecting venules. Wild-type (open circles) and CD43-deficient (filled circles) lymphocytes that established a primary tether in order I and II venules without prior interaction in upstream HEV were counted. The tethering fraction was determined as the percentage of newly tethered cells in the total number of cells passing through that venule during the observation period. Data pairs connected by solid lines indicate tethering fractions of both lymphocyte samples in the same venule; filled squares connected by a dashed line indicate average tethering fractions. The difference in mean tethering frequencies was highly significant ($P < 0.001$, Student's t test for paired means; $n = 7$ venules in three animals).

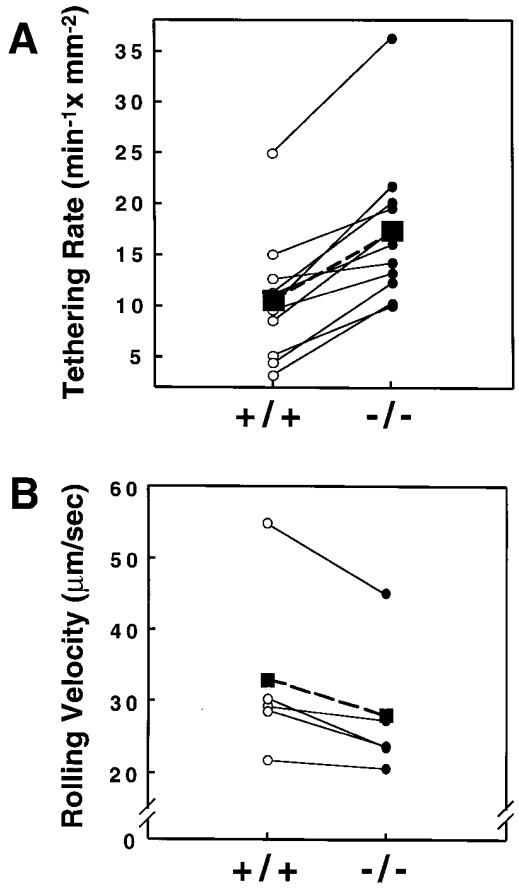

Figure 6. Increased Tethering and Slower Rolling of CD43-Deficient Lymphocytes In Vitro

(A) Tethering rates of lymphocytes to immobilized PNAd were determined in a flow chamber (1.6 dyn/cm^2) for CD43$^{-/-}$ (filled circles) and wild-type (open circles) cells. Data pairs connected by solid lines indicate tethering fractions of both lymphocyte samples in the same experiment; filled squares connected by a dashed line indicate average tethering fractions. The difference in mean tethering frequencies was highly significant ($P < 0.001$, Student's t test for paired means).

(B) The mean rolling velocities of 50 consecutive wild-type (open circles) and CD43$^{-/-}$ (filled circles) lymphocytes were determined in identical fields in five independent experiments. Data pairs connected by solid lines indicate mean rolling velocities of both lymphocyte samples in the same experiment; the dashed line connects average rolling ratios (filled squares). The difference in rolling velocities was modest but statistically significant ($P < 0.05$, Student's t test for paired means).

might provide direct steric hindrance of L-selectin engagement with a surface-bound ligand. On the other hand, lymphocyte-expressed L-selectin bound a freely diffusible ligand, PPME-FITC, equivalently, whether CD43 was present or not. This suggests that CD43 functions primarily by restricting the accessibility of L-selectin, but not its function.

Based on these considerations, we predict that the homing efficiency of lymphocytes might be regulated in part by alterations in the surface expression of CD43. It has been shown that the sialylation of CD43 is regulated in resting and activated T cells (Piller et al., 1988), and that upon activation, CD4$^+$ T cells express hyposialylated CD43 that carries a reduced charge (Jones et al., 1994). In addition, CD43 molecules are excluded from contact areas between activated monocytes and immunoglobulin-coated erythrocytes (Soler et al., 1997), and neutrophils enzymatically cleave the extracellular portion of CD43 upon stimulation (Remold-O'Donnell and Parent, 1994). Such a charge reduction might limit the barrier effect on cell adhesion imparted by CD43, thus allowing increased interactions of activated leukocytes with endothelial cells and/or other targets, particularly in vascular beds with limited expression of pro-adhesive ligands. Conversely, for immunologically naive T cells

that express the extensively sialylated form, CD43 expression may prevent promiscuous adhesion in vascular beds where ligand density is too low to override its anti-adhesive effect. The net result of anti-adhesion could be enhanced selectivity of naive T cell migration to lymphoid organs that display the highest concentration of homing receptor ligands, thereby channeling T cells to those sites that are most likely to contain their cognate antigen.

In conclusion, we demonstrate that a surface-expressed molecule, CD43, can negatively regulate T cell homing in vivo by attenuating engagement of the lymph node homing receptor, L-selectin. We propose that the remarkable specificity of lymphocyte migration is determined not simply by molecular events that confer mechanical stability to cell-to-cell interactions, but is the result of a finely tuned equilibrium of pro- and anti-adhesive mechanisms.

Experimental Procedures

Antibodies

Antibodies to the murine adhesion molecules L-selectin (mAb Mel-14), α4 integrin (mAb PS/2), the α chain of LFA-1 (mAb Tib 213), and PNAd (mAb MECA-79) were purified from culture supernatants. mAbs to CD44 (mAb Tjb 1.7) and β7 integrins (mAb Fib 504) were kindly provided by Eugene Butcher. Anti-CD43 (mAb S7) was a generous gift from Tom Waldschmidt. All mAbs were stored in endotoxin-free phosphate-buffered saline (PBS) at -70°C until use. Phycoerythrin (PE)–conjugated anti-Thy1.2 was purchased from PharMingen (San Diego, CA). mAb MECA-79 was dialyzed against bicarbonate buffer (pH 8.2) and fluorescently labeled by addition of 25 μg of carboxy fluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) per milligram of protein. After incubation at room temperature for 1 hr, the reaction was stopped by dialysis against PBS. Aliquots of mAbs Mel-14 and S7 were biotinylated for flow cytometry (described below). PE-conjugated streptavidin (PharMingen) was used as a second step for biotinylated mAbs; PE-labeled goat anti-rat-immunoglobulin $F(ab)_2$ (PharMingen) was used to detect other mAbs where appropriate.

Homing Assays

Wild-type and CD43$^{-/-}$ mice were euthanized to prepare single-cell suspensions of PLN lymphocytes. First, 5×10^7 cells of each population were mixed and injected through a catheter in the right jugular vein of anesthetized AKR/J recipients. Cells remaining in the catheter were saved to control for potential differences in cell input. After 2 hr, recipients were again anesthetized and exsanguinated by puncture of the retroorbital plexus. Peripheral blood lymphocytes were isolated by density gradient separation on Lympholyte-M (Cedar Lane, Ontario, Canada). Spleen, PLN, MLN, PP, and lungs of recipients were dissected and pressed through fine wire mesh to yield single-cell suspensions. Isolated cells were placed in 96-well V-bottom plates (Costar, Cambridge, MA) at 1–5 $\times$ 10^6 cells/well and double-stained with PE-conjugated anti-Thy1.2 and biotinylated anti-CD43 mAb S7 followed by streptavidin-FITC.

Flow Cytometry

Single-cell suspensions were analyzed on a flow cytometer (Becton Dickinson) after gating for viable lymphocytes by forward and light scatter characteristics. For detection of cells from wild-type and CD43$^{-/-}$ donors, the relative frequency of Thy1.2$^+$ CD43$^+$ and Thy1.2$^+$ CD43$^-$ donor T cells was determined in peripheral blood, lymphoid organs, and lungs of AKR/J recipients by two-color flow cytometry. The relative frequency of the two donor cell populations was determined for each individual organ and corrected for differences in cell input in the same animal. Control experiments confirmed that AKR/J lymphocytes did not express the Thy1.2 epitope (data not shown), and essentially all Thy1.2-positive wild-type lymphocytes expressed the S7 antigen (Gulley et al., 1988), but none of the CD43$^{-/-}$ cells was reactive with S7. Adhesion receptor expression on isolated PLN lymphocytes was analyzed using biotinylated mAbs Mel-14 and S7 and unconjugated mAbs Tjb 1.7, PS/2, Fib504, and Tib 213 followed by appropriate second-stage reagents.

PPME-FITC Binding Assay

PLN lymphocytes from from four pairs of age- and sex-matched wild-type and CD43$^{-/-}$ littermates were suspended in Dulbecco's modified Eagle's medium containing 40 mM HEPES and 10% fetal bovine serum (assay media). Cells were aliquoted (3×10^5 cells/tube) and kept on ice for 30 min in the presence of assay media with or without 25 mM EDTA or 25 μg/ml Mel-14. Subsequently, PPME-FITC was added to all tubes to a final dilution of 1:1000. Control experiments with this batch of PPME-FITC had determined that at this dilution PPME-FITC binding is specific for L-selectin, since it was nearly completely blocked by EDTA or mAb Mel-14. After 30 min on ice, samples were analyzed by flow cytometry without prior washing or fixing of cells. The mean fluorescence intensity of PPME-FITC–labeled cells was determined after acquisition of 5000 events on a linear fluorescence scale, and specific binding was calculated by subtracting background staining (i.e., mean fluorescence intensity in the presence of mAb Mel-14). In addition, aliquots of each lymphocyte sample were stained with biotinylated mAb Mel-14 followed by FITC-streptavidin. In parallel, the same labeling protocol was used to stain standardized microbeads, permitting the quantitation of the mean number of L-selectin molecules per lymphocyte (Quantum Simply Cellular, Flow Cytometry Standards, San Juan, Puerto Rico). The L-selectin lectin activity of wild-type and CD43-deficient cells was expressed as the mean fluorescence intensity of specific PPME-FITC binding per 100,000 L-selectin molecules.

PLN Histology

Axillary, brachial, cervical, and inguinal PLN from age- and sex-matched wild-type and CD43 deficient donors (30 PLN from four animals in each group) were harvested, fixed in formalin (10% in PBS), embedded in paraffin, serially sectioned, and stained with hematoxilin–eosin. Sections through the central plane of each PLN (i.e., those containing the largest cross-sectional area) were photographed, and cross-sectional areas of PLN were measured by computer-assisted planimetry.

Intravital Microscopy

Intravital microscopy of PLN microvessels and lymphocyte behavior in the venular tree has been described in detail elsewhere (Diacovo et al., 1996; von Andrian, 1996; Warnock et al., 1998). In brief, young adult C57BL/6 $\times$ 129Sv/J1 wild-type animals were anesthetized by intraperitoneal injection of 5 mg/ml ketamine and 1 mg/ml xylazine (10 ml/kg) and surgically prepared for microscopic observation of the left subiliac lymph node (von Andrian, 1996). Catheters were inserted in the right femoral artery, the left carotid artery, and the right jugular vein. Subsequently, the preparation was transferred to an intravital microscope (IV-500, Mikron Instruments, San Diego, CA). Lymphocytes from PLN of wild-type and CD43$^{-/-}$ donor animals were fluorescently labeled with 2′,7′,-bis-(2-carboxyethyl)-5(and $-$6) carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) and retrogradely injected through the femoral artery catheter. Cells were observed by stroboscopic epifluorescence illumination through $\times$10 or $\times$20 water-immersion objectives (Achroplan, Zeiss, Germany) and recorded on Hi8 video tape using a silicon-intensified target camera (VE-1000 SIT, Dage mti, Michigan City, IN). To control for possible changes in PLN during the course of an experiment, the order of lymphocyte sample injections was alternated between experiments.

Video Analysis

Lymphocyte behavior in PLN venules was analyzed essentially as described (von Andrian, 1996; Warnock et al., 1998). In brief, rolling fractions were assessed by determining the percentage of lymphocytes that interacted with the venular lining in the total flux of cells that passed the same venule during an observation period. Rolling cells that became subsequently stuck were included in the rolling fraction. In some experiments, the rolling distance was measured

by tracing individual cells that began to roll in order IV or V venules until they detached. Only cells that did not stick during their passage through PLN were included in this analysis. Lymphocyte tethering was determined in order I and II collecting venules. To do so, we determined the frequency of injected cells that initiated rolling in a venule without prior interactions in an upstream vessel. A "sticker" was defined as a cell that arrested in a perfused venule and did not move for at least 30 seconds. Two different parameters for sticking were obtained: stickers/rollers (percentage) is the number of stickers per 100 rolling cells; stickers/total (percentage) is the percentage of stickers in the total (i.e., rolling plus noninteracting) flux of cells. The former parameter assesses the ability of a cell population to stick independent of its ability to undergo primary rolling interactions; the latter (termed "sticking fraction") is a measure of the frequency of injected cells that can undergo the entire three-step adhesion cascade as defined previously (Warnock et al., 1998).

MECA-79 Antigen Localization in PLN

CFSE-conjugated mAb MECA-79 (50 μg in 300 μl of saline) was injected through the femoral artery catheter, and fluorescent mAb localization in the contralateral subiliac PLN was recorded on videotape 10 min later with the aid of a silicon-intensified target camera with adjustable gain and black level settings (VE1000-SIT, Dage mti). The venular tree of PLN was visualized through a ×4 dry objective (Zeiss Achroplan, Germany) and videotaped for assignment of branching order designations as described (von Andrian, 1996). Higher-power water-immersion objectives (×10, ×20, and ×40) were used to assess mAb MECA-79 accumulation in individual venules.

Flow Chamber Assays

PNAd was immunopurified from human tonsils and coated on a plastic slide to study tethering and rolling of murine lymphocytes in a flow chamber apparatus as described (Diacovo et al., 1996). Wild-type and CD43$^{-/-}$ PLN lymphocytes were perfused over immobilized PNAd at 1×10^6 cells/ml at a constant shear stress (1.6 dyne/cm^2) and recorded on videotape. The number of tethered cells was counted during a 3 min period after the frequency of rolling had reached equilibrium. Infusions of assay media containing 5 mM EDTA were used to clear the field of view of all adherent cells between recordings. Video recordings of flow chamber experiments were also analyzed to measure rolling velocities of 50 consecutive wild-type or mutant cells using a computer–based image analysis system (Pries, 1988). Wild-type and CD43$^{-/-}$ cells were compared while rolling through the identical field of view. Lymphocytes that displayed erratic rolling behavior (skipping or a rolling distance of less than three cell diameters) were not included.

Acknowledgments

We thank Eugene Butcher for generously providing hybridomas for mAbs Mel-14 and MECA-79 and purified mAbs Tjb1.7 and FIb504 and Tom Waldschmidt for kindly supplying mAb S7. We are grateful to Lloyd Stoolman for providing PPME-FITC. We thank Thomas Diacovo for advice on flow chamber studies, Brian P. Fors for technical assistance, and Ellen Winchester for assistance with morphometric analyses. This work was supported by National Institutes of Health grants HL54936, HL56949, AI40225, and AI41710, and a grant from Biosource Technologies (Vacaville, CA).

Received December 24, 1997.

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