Brief Definitive Report

The Functional Paradox of CD43 in Leukocyte Recruitment: A Study Using CD43-deficient Mice

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Summary

Although there is considerable evidence implicating a role for CD43 (leukosialin) in leukocyte cell–cell interactions, its precise function remains uncertain. Using CD43-deficient mice (CD43$^{-/-}$) and intravital microscopy to directly visualize leukocyte interactions in vivo, we investigated the role of CD43 in leukocyte–endothelial cell interactions within the cremasteric microcirculation under flow conditions. Our studies demonstrated significantly enhanced leukocyte rolling and adhesion after chemotactic stimuli in CD43$^{-/-}$ mice compared with wild type mice. Using an in vitro flow chamber, we established that the enhanced rolling interactions of CD43$^{-/-}$ leukocytes, primarily neutrophils, were also observed using immobilized E-selectin as a substrate, suggesting that passive processes related to steric hindrance or charge repulsion were likely mechanisms. Despite increased adhesion and rolling interactions by CD43$^{-/-}$ leukocytes, we uncovered a previously unrecognized impairment of CD43$^{-/-}$ leukocytes to infiltrate tissues. Oyster glycogen–induced neutrophil and monocyte infiltration into the peritoneum was significantly reduced in CD43$^{-/-}$ mice. In response to platelet activating factor, CD43$^{-/-}$ leukocytes were impaired in their ability to emigrate out of the vasculature. These results suggest that leukocyte CD43 has a dual function in leukocyte–endothelial interactions. In addition to its role as a passive nonspecific functional barrier, CD43 also facilitates emigration of leukocytes into tissues.

Key words: CD43 • leukocytes • recruitment • antiadhesive • emigration

There is now considerable evidence demonstrating that the recruitment of leukocytes from the circulation to sites of tissue injury or inflammation is mediated by a sequential cascade of leukocyte–endothelial cell interactions (1). Tethering and rolling are the first interactions that occur between circulating leukocytes and endothelial cells. Rolling leukocytes can be activated to firmly adhere to the vascular endothelium, and subsequently emigrate between endothelial cells into the extravascular space. Each of these events is known to be mediated by distinct classes of adhesion molecules; selectins are responsible for leukocyte tethering and rolling, while β_2 integrins and members of the immunoglobulin superfamily mediate adhesion and emigration. In contrast, very little is known about existing antiadhesive mechanisms that may regulate or attenuate the inflammatory response. A functional barrier that prevents leukocyte–endothelial cell interactions through either steric hindrance or charge repulsion may be one potential mechanism.

One such barrier may be provided by the cell-surface sialoglycoprotein CD43 (leukosialin), a molecule expressed exclusively on hematopoietically derived cells (2, 3). There are several compelling reasons to consider that CD43 may have an important role in cell–cell interactions. First, its structure is consistent with a barrier inasmuch as the extracellular domain of CD43 is extraordinarily long and extends 45 nm from the plasma membrane (4). Through steric hindrance, CD43 may interfere with the ability of other adhesion molecules, including L-selectin (5), to interact with their ligands. Second, CD43 has abundant sialic acid residues that impart a net negative surface charge thought to retard cell–cell interactions (6). Desialation of neutrophils has been associated with a reduction in cell-surface charge and increased adhesiveness, homotypic aggregation, and cell-spreading (6, 7). Third, CD43 is partially downregulated by proteolytic shedding after cellular activation (6, 8) perhaps exposing adhesion molecules and reducing repulsive forces.

Studies using an antibody directed against CD43 do not support the view that CD43 is antiadhesive—in fact this antibody appears to reduce the recruitment of leukocytes into tissues (9, 10). Although it is conceivable that anti-CD43 antibodies could paradoxically enhance barrier func-

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tion by increasing steric hindrance, it is also possible that CD43 functions as a homing receptor promoting leukocyte recruitment into tissues or as an accessory molecule enhancing leukocyte rolling, adhesion, and emigration. Using the CD43-deficient ($CD43^{-/-}$) mouse described by Manjunath et al. (11) and intravital microscopy to visualize leukocyte kinetics in vivo (12), we directly visualized and investigated the function of CD43 in leukocyte–endothelial cell interactions within the cremasteric microcirculation under flow conditions. Our results demonstrate that leukocyte rolling and adhesion induced by chemotactic stimuli are enhanced in $CD43^{-/-}$ mice compared with wild-type control animals, but unexpectedly there is an inability of $CD43^{-/-}$ leukocytes (namely neutrophils and monocytes) to emigrate out of the vasculature.

Materials and Methods

Animals. CD43-deficient mice ($CD43^{-/-}$) produced by homologous recombination in embryonic stem cells were generated in a mixed background of 129/SvEv × C57BL/6 as previously described (11), and were obtained from The Jackson Laboratory (Bar Harbor, ME). Wild-type mice derived from the same background were used as controls. Animals were bred and housed in specific pathogen-free facilities and used between 6 and 12 wk of age.

Mouse Cremaster Preparation. The mouse cremaster preparation was used to investigate leukocyte–endothelial cell interactions in the microcirculation (12). Mice were anesthetized by intraperitoneal injection of a mixture of xylazine hydrochloride (10 mg/kg; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and ketamine hydrochloride (200 mg/kg; Rogar/STB Inc., London, Ontario, Canada). The jugular vein was cannulated and used to maintain anesthesia. The cremaster muscle was dissected free of tissues and exteriorized onto an optically clear viewing pedestal. The muscle was cut longitudinally with a cautery and held flat against the pedestal by attaching silk sutures to the corners of the tissue. The muscle was then superfused with bicarbonate-buffered saline warmed to 37°C.

An intravital microscope (Axioskop, Carl Zeiss Inc. Canada, Don Mills, Ontario, Canada) has been described elsewhere. Single unbranched cremasteric postcapillary venules (25–40 μm in diameter) were selected for examination of leukocyte rolling and adhesion. Leukocytes were considered adherent to the venular endothelium if they remained stationary for a period ≤30 s. Rolling leukocytes were defined as those moving at a velocity less than that of erythrocytes within a given vessel. Leukocyte rolling velocity was calculated from the time taken for a leukocyte to roll 100 μm. Venular diameter (D_v) was measured on-line using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). Centerline red blood cell velocity (V_{RBC}) was also measured on-line using an optical Doppler velocimeter (Microcirculation Research Institute) and mean red blood cell velocity (V_{mean}) was determined as $V_{RBC}/1.6$. Venular wall shear rate was calculated based on the Newtonian definition: $\gamma = 8\ (V_{mean}/D_v)$ (13).

Experimental Protocol. To determine whether CD43 was capable of regulating leukocyte recruitment induced by an acute chemotactic stimulus, the response to the bacterial peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma Chemical Co., St. Louis, MO) was examined. fMLP (10 μM) was superfused over the cremasteric preparations for 60 min and leukocyte rolling and adhesion in postcapillary venules determined at 15-min intervals.

Leukocyte Emigration. To examine whether leukocyte emigration was impaired in $CD43^{-/-}$ mice, emigration induced by platelet-activating factor (PAF), a potent promoter of leukocyte emigration in the mouse cremaster microcirculation, was studied (14). In this final group of intravital microscopy experiments, cremasteric preparations were superfused for 60 min with 100 nM PAF in a saline solution containing 0.5% BSA. PAF was used because fMLP did not induce emigration in the murine system.

Peritoneal Elicitation. Mice were given a 1-ml i.p. injection of 1% oyster type II glycogen in sterile saline as previously described (15). After 4 or 24 h, cells were harvested from the peritoneal cavity by lavage via 3 ml of sterile saline, and then counted using a hemocytometer. Differential counting was performed with Wright-Giemsa staining. Finally, to ensure that incomplete harvest was not responsible for lower leukocyte yields in the peritoneum of $CD43^{-/-}$ mice, peritoneums were also lavaged with heparin and EDTA. Similar results were obtained with heparin or EDTA.

Flow Chamber Assay. To study murine leukocyte behavior under shear conditions in vitro, whole blood was perfused over immobilized E-selectin (5 μg/ml), using a previously described flow chamber assay (12). Coverslips were mounted into a polycarbonate chamber with parallel plate geometry (16) and observed at a magnification of 100 using an inverted microscope equipped with phase-contrast optics (Carl Zeiss Inc. Canada). The stage area was enclosed in a warm air cabinet and maintained at 37°C.

In brief, the blood was diluted 1:10 in HBSS, maintained at 37°C using a water bath, and perfused through the flow chamber at defined wall shear stresses using a syringe pump (Harvard Apparatus Inc., South Natick, MA). All of the experiments described were performed between 2 and 4 dynes/cm^2. The blood was perfused over the substrate for 3 min and then chased with HBSS to flush out any remaining erythrocytes and noninteracting leukocytes. At this stage leukocytes interacting with the coverslip could be seen readily and were counted in at least four random fields per coverslip and expressed as the number of interacting cells per field of view.

Statistical Analysis. All data are presented as mean ± SEM. The data within groups were compared using a paired Student's *t* test using Bonferroni corrections for multiple comparisons as required. Unpaired *t* tests were used to compare between groups. Statistical significance was set at $P < 0.05$.

Results and Discussion

Leukocyte Interactions in Single Microvessels of $CD43^{-/-}$ Mice. In vivo fMLP (10 μM) superfusion over the cremasteric microcirculation revealed a significant difference in leukocyte kinetics between wild-type ($CD43^{+/+}$) and $CD43^{-/-}$ mice (Fig. 1). Over the first 60 min of fMLP superfusion, the number of rolling cells in $CD43^{-/-}$ animals was maintained at a constant level and was significantly greater in comparison with wild-type mice ($P < 0.05$). Moreover, this higher level of rolling was associated with a significant augmentation in leukocyte adhesion (31.3 ± 2.8 in $CD43^{-/-}$ vs. 13.3 ± 2.0 in $CD43^{+/+}$; $n = 7$ in each group; $P < 0.05$). CD43 deficiency did not influence leukocyte rolling velocity.

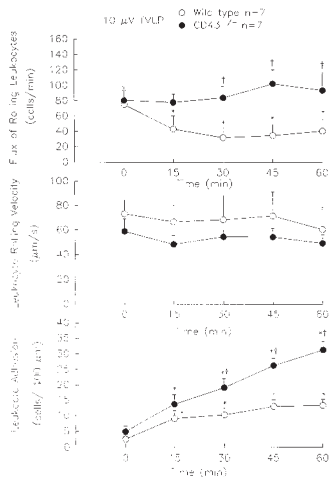

Figure 1. The flux of rolling leukocytes (*top*), leukocyte rolling velocity (*middle*), and leukocyte adhesion (*bottom*) in CD43$^{-/-}$ (n = 7) and CD43$^{+/+}$ (n = 7) mice during superfusion of the cremasteric microcirculation with 10 μM fMLP. Leukocyte rolling and adhesion were examined at 15-min intervals over 1 h. Both leukocyte rolling and adhesion were enhanced in the CD43$^{-/-}$ mice compared with wild-type, while rolling velocity was unaffected. $^*P < 0.05$ relative to 0 min. $^{\dagger}P < 0.05$ relative to respective CD43$^{-/-}$ value.

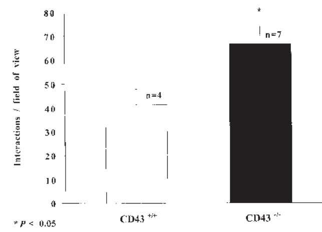

Figure 2. Leukocyte interactions (rolling and adhesion) per field of view after perfusion of whole blood from CD43$^{-/-}$ (n = 7) and CD43$^{+/+}$ (n = 4) mice over immobilized E-selectin in vitro under flow conditions. Leukocytes from the CD43$^{-/-}$ mice had significantly more (60%) interactions (66.5 ± 7.4) compared with leukocytes from the CD43$^{+/+}$ mice (41.2 ± 6.6). Light microscopy demonstrated that >85% of the leukocytes were neutrophils (data not shown). $^*P < 0.05$.

These results demonstrate that CD43$^{-/-}$ leukocytes have an enhanced ability to interact with the endothelium of postcapillary venules in vivo after fMLP stimulation. Direct visualization of leukocyte–endothelial cell interactions revealed a nonselective effect in that both rolling and adhesion were enhanced in CD43$^{-/-}$ mice after exposure to fMLP. fMLP directly activates neutrophils to adhere via CD18–ICAM-1 interactions (17, 18), and our data demonstrate that in the absence of CD43 this interaction was far more avid. Although this is the first documentation of enhanced adhesion in peripheral microvessels in vivo, it is consistent with an earlier in vitro report demonstrating that CD43 deficiency can enhance lymphocyte binding to ICAM-1 and fibronectin (11), and that HeLa cells demonstrate reduced LFA-1–ICAM-1 interactions after CD43 transfection (19). Although an initial report suggested that CD43 may be proadhesive in that it functions as a ligand for ICAM-1 (20), this observation has not been confirmed by our study in vivo or by others in vitro (9, 10). The most compelling evidence that CD43 does not function as a ligand for ICAM-1 is that leukocytes from CD18-deficient (leukocyte adhesion deficiency type 1) patients with ample CD43 did not bind to ICAM-1 (21, 22). However, this does not exclude the possibility that CD43 could be an accessory molecule and not a direct ligand for ICAM-1.

Leukocyte rolling is dependent upon activation-regulated selectins (E and P) on the endothelium (1). To exclude the possibility that enhanced leukocyte rolling observed in vivo in CD43$^{-/-}$ animals was due to enhanced upregulation of endothelial selectins compared with wild-type animals, we compared the ability of CD43$^{+/+}$ and CD43$^{-/-}$ leukocytes to interact with immobilized (unalterable) E-selectin in vitro under flow conditions. Fig. 2 demonstrates enhanced interactions of CD43$^{-/-}$ leukocytes in vitro when whole blood was perfused over immobilized E-selectin. Light microscopy of the coverslips for both wild-type and CD43$^{-/-}$ leukocytes revealed that >85% of the interacting cells were neutrophils (data not shown). Differences in circulating leukocyte counts between CD43$^{-/-}$ and CD43$^{+/+}$ mice do not explain this result. Peripheral blood total leukocyte counts and differentials were similar in both animal groups, which is consistent with an earlier report (11). These results suggest that the difference in rolling between CD43$^{-/-}$ and CD43$^{+/+}$ neutrophils was a passive process perhaps related to charge or steric hindrance rather than a specific activation or deactivation event associated with CD43$^{-/-}$ mice. Indeed, recent work suggested ~50% increased tethering of CD43$^{-/-}$ lymphocytes to L-selectin ligands in vivo and in vitro entirely consistent with the view that CD43 indiscriminately inhibits leukocyte interactions via all three selectins: P-selectin and E-selectin in this study and L-selectin in the work of Stockton et al. (5). Together the evidence suggests that CD43 deficiency may have a similar effect on most, if not all, leukocytes.

The molecules that support leukocyte rolling (L-selectin, P-selectin glycoprotein ligand–1, and α4-integrin) have been typically localized to sites on microvilli (23–25), and may still be able to interact with ligands despite the presence of CD43. Indeed, our own data clearly demonstrated that CD43$^{+/+}$ leukocytes rolled effectively on all of the substrata studied albeit not as effectively as CD43$^{-/-}$ cells. An explanation for this difference may be that the negatively charged sialic acid residues on CD43 also contribute to the functional barrier in an electrochemically repulsive manner rather than just by steric hindrance. This is consistent with the observation that desialation of neutrophils has been associated with a reduction in cell-surface charge and an increase in adhesiveness (6, 7).

Studies using mAbs have not confirmed an antiadhesive role for CD43. In several studies, cross-linking of CD43 by antibodies induces leukocyte aggregation (19, 22, 26–29) possibly through the regulation of integrin function (22, 29, 30), whereas in other studies anti-CD43 antibodies have been shown to prevent leukocyte adhesion (9, 10) and downregulate leukocyte integrin expression (31). Recently, an anti-CD43 antibody (L11) was shown to reduce neutrophil and monocyte recruitment into the peritoneal cavity (10) as well as block T cell homing into secondary lymphoid tissues (9). However, based on our results with enhanced cell adhesion to endothelium, we reasoned that CD43$^{-/-}$ animals would recruit neutrophils into the peritoneum at least as well as CD43$^{+/+}$ mice. Surprisingly, i.p. injection of 1% oyster glycogen did not produce the anticipated results (Fig. 3 *A*). CD43$^{-/-}$ animals had a significant

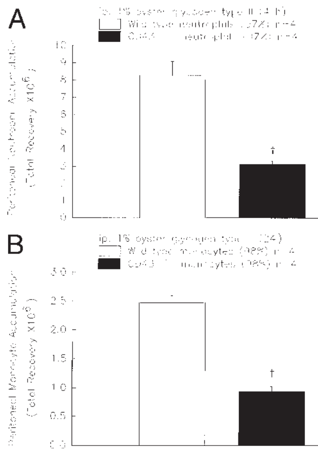

Figure 3. Leukocyte emigration in CD43$^{-/-}$ and CD43$^{+/+}$ mice 4 h (*A*) and 24 h (*B*) after a 1-ml i.p. injection of 1% oyster glycogen (type II). Leukocytes were harvested by lavage with sterile saline and counted using a hemocytometer. CD43$^{-/-}$ mice had a significant 50% reduction in leukocyte recruitment compared with CD43$^{+/+}$ mice. Light microscopy demonstrated that >97% of the leukocytes isolated at 4 h were neutrophils and >98% were monocytes at 24 h. $^{\dagger}P < 0.05$.

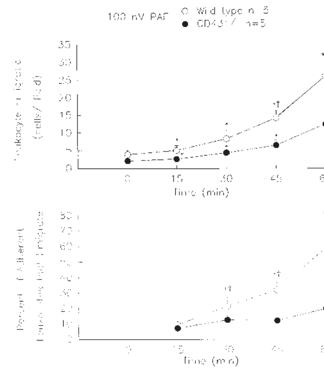

Figure 4. Leukocyte emigration (*top*) during superfusion of the cremasteric microcirculation with 100 nM of PAF for 60 min. Emigration was examined at 15-min intervals over 1 h. The percentage of adherent leukocytes that emigrated in response to PAF is shown. The CD43$^{-/-}$ mice ($n = 5$) had a significant impairment in the emigration of adherent leukocytes compared with CD43$^{+/+}$ mice ($n = 5$). $^{*}P < 0.05$ compared with 0 min. $^{\dagger}P < 0.05$ relative to respective CD43$^{+/+}$ value.

impairment (≥50%) in leukocyte recruitment into the peritoneal cavity at 4 h ($P < 0.05$). Cell counting revealed that >97% of the cells isolated were neutrophils in both groups of animals (data not shown). When these same experiments were performed for 24 h very similar results were observed (Fig. 3 *B*). More than a twofold increase in leukocytes was noted in wild-type animals but at this time 98% of the cells were mononuclear leukocytes.

These results are consistent with those of McEvoy et al., in which anti-CD43 antibodies blocked neutrophil recruitment into the peritoneum (10). However, the lack of recruitment could be due to several mechanisms including enhanced neutrophil–neutrophil interactions within the vasculature, increased apoptosis, and macrophage-mediated clearance of neutrophils within the peritoneum, or impaired emigration. To resolve this issue we elicited leukocyte recruitment out of the cremasteric microvasculature so that we could directly visualize leukocyte emigration into tissues. Because fMLP did not elicit a significant increase in leukocyte emigration, we superfused the cremaster with PAF. In response to 60 min of optimal concentrations of PAF (100 nM) (14), the CD43$^{-/-}$ animals had a striking, >50% reduction in leukocyte emigration (13.3 ± 2.0 per field of view) compared with CD43$^{+/+}$ animals (31.3 ± 2.8 per field of view). These results demonstrate for the first time a defect in neutrophil emigration associated with CD43 deficiency. When the proportion of adherent leukocytes that emigrated was determined for each group (Fig. 4), CD43$^{-/-}$ leukocytes clearly had a significant impairment in emigration. In response to 60 min of PAF, only 20 ± 6% of the adherent CD43$^{-/-}$ leukocytes emigrated whereas 59 ± 17% of the adherent CD43$^{+/+}$ leukocytes emigrated ($n = 5$ each group; $P < 0.01$). Preliminary work from our laboratory suggest that leukocyte movement across semipermeable membranes was reduced three-fold in the absence of CD43$^{-/-}$ (Woodman, R.C., and P. Kubes, unpublished observation). These results strongly suggest that CD43 may have an additional function besides acting simply as a passive barrier. This may explain the paradox between increased adhesion of CD43$^{-/-}$ leukocytes in vitro on one hand but an inability in vivo of leukocytes from CD43-deficient animals to effectively emigrate into tissues as well as clear viral infections (11). It is possible that enhanced adhesive interactions associated with CD43 deficiency may prevent the sequential attachment and detachment required for the leukocyte emigration.

Based on this series of experiments, our results strongly support the view that CD43 functions to inhibit leukocyte–endothelial cell interactions by limiting rolling and adhesion within the microvasculature. However, once a leukocyte is adherent, CD43 clearly functions to enhance emigration out of the vasculature; complete absence of this molecule leads to a dramatic impairment in leukocyte infiltration into tissues. It is well known that CD43 is proteolytically shed from the surface of phagocytic cells upon activation (6, 8) and that downregulation of CD43 occurs with leukocyte emigration into tissues (32, 33) but, interestingly, there is never complete shedding. In fact, ~50% of the CD43 remains membrane bound even after prolonged exposure to chemotactic factors such as PAF (34). It is conceivable that the remaining CD43 on an adherent leukocyte functions to either signal leukocyte emigration out of the vasculature thus contributing to the emigration process, or reduce leukocyte–endothelial interactions during transmigration. Alternatively, CD43 may repel newly adherent leukocytes which might otherwise bind to the emigrating cell and impair its progress across the endothelium.

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