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CD43 Deficiency Has No Impact in Competitive In Vivo Assays of Neutrophil or Activated T Cell Recruitment Efficiency

Douglas A. Carlow2 and Hermann J. Ziltener2

Using noncompetitive methodologies comparing CD43+/+ and CD43−/− mice, it has been reported that CD43−/− leukocytes exhibit reduced recruitment efficiency to sites of inflammation. More recent analyses demonstrate that CD43 on activated T cells can function as an E-selectin ligand (E-SelL) in vitro, suggesting that CD43 might promote rolling interactions during recruitment of leukocytes and account for the reported recruitment deficits in CD43−/− T cells and neutrophils in vivo. Internally controlled competitive in vivo methods using fluorescent tracking dyes were applied to compare recruitment efficiency of CD43+/+ vs CD43−/− activated T cells to inflamed skin and of peripheral blood neutrophils to inflamed peritoneum. A simple CFSE perfusion method was developed to distinguish arterial/venous vasculature and confirm appropriate extravasation through venules in a Con A-induced cutaneous inflammation model. In vivo recruitment of peripheral blood neutrophils to inflamed peritoneum was core 2 GlcNAcT-I dependent, but recruitment efficiency was not influenced by absence of CD43. There were also no significant differences in core 2 GlcNAcT-I-dependent, selectin-dependent, cutaneous recruitment of activated T cells from CD43+/+ and congenic CD43−/− mice in either B6 or P-selectin−/− recipients despite biochemical confirmation that a CD43-specific E-SelL was present on activated T cells. We conclude that recruitment of neutrophils and activated T cells in these in vivo models is not influenced by CD43 expression and that if CD43 on activated T cells performs an E-SelL function in vivo, it contributes in a limited physiological context. The Journal of Immunology, 2006, 177: 6450–6459.

During the inflammatory response, various cell types, including T cells and neutrophils, exit the vasculature and accumulate within inflamed tissue in a process collectively referred to as recruitment (1). Recruitment generally starts with rolling behavior of cells expressing selectin ligands along venule endothelia that express P- and E-selectin homing receptors (1, 2). Both E- and P-selectins are induced on inflamed endothelium in most tissues (3, 4), but constitutively expressed on skin endothelium in mice (5). The primary P-selectin glycoprotein ligand 1 (PSGL-1) is only functional after appropriate (including core 2 GlcNAcT-I (C2)) modifications of O-linked carbohydrates (6, 7) that are constitutively present in myeloid cells (8) and inducible in T cells (9, 10). E-selectin ligands (E-SelL), some requiring similar O-glycan modifications, are thought to include PSGL-1 (11, 12), but additional E-SelL, such as ESL-1, CD66, CD44, α-2-3-sialyl Lewis x glycosphingolipids, and β2 integrin, may also be active. Whether any of these alternate E-SelL contribute to physiologically relevant E-SelL functions is not clear.

Recent data based on in vitro observations of murine (13) and human (14) T cells have identified the activation-associated glycoform of CD43 as a potential novel ligand for E-selectin. These observations suggest that under some conditions, CD43 might facilitate rolling interactions and function as a homing receptor during E-selectin-mediated recruitment. The proposal that CD43 might function as an E-SelL was particularly surprising given previous work using intravital microscopy (IVM) describing a higher, not lower, frequency of rolling neutrophils and monocytes on activated venules in mice lacking CD43; notably, CD43−/− leukocytes also exhibited retarded transmigration that collectively resulted in a net >2-fold reduction in recruitment (15). In terms of net consequences on recruitment, these observations found some support in reports in which in vivo T cell homing to secondary lymphoid organs (16) and recruitment to inflammatory sites (17) were inhibited with an Ab to CD43. In models of lymphocytic choriomeningitis virus (LCMV) infection and experimental autoimmune encephalomyelitis induction, CD43−/− CD8 T cells (18) and CD4 T cells (19), respectively, exhibited reduced CNS recruitment.

In an effort to directly assess the influence of CD43 during recruitment, we have developed in vivo competitive methods to compare recruitment efficiencies of two or more cell populations at an inflammatory site within a single mouse. Such assays help eliminate the influence of environmentally rooted differences in the mouse strains being compared and indirect effects of a null mutation, transgene, or treatment on the organism that might affect recruitment (1). Using available fluorescent tracking dyes to mark competing cell populations, competitive in vivo recruitment assays have single-cell resolution, and thus the potential for sensitive quantitative analysis, even when recruited cell numbers are small.

We applied these competitive in vivo recruitment methods to assess efficiency of selectin ligand-dependent recruitment of control CD43+/+ vs CD43−/− activated T cells to cutaneous inflammation and of peripheral blood neutrophils (PBN) to inflamed peritoneum. Lack of CD43 expression had no detectable impact on recruitment of activated T cells to inflamed skin, even in the absence of P-selectin, conditions in which recruitment was expected...
to be highly E-selectin dependent. Similarly, C2-dependent recruitment of CD43-bearing and CD43-deficient PBN to inflamed peritoneum occurred with equal efficiency. Despite confirmation that the activation-associated glycoform of CD43 on activated T cells can perform as an E-SeleL in vitro, CD43 deficiency had no detectable influence on recruitment in the two in vivo competitive recruitment models described. Our results suggest that the abundance of CD43-associated E-SeleL on activated T cells is too low to be functionally relevant for recruitment.

**Materials and Methods**

**Mice**

Mice aged 8–20 wk were used for analyses. C57BL/6 (B6) mice were bred at the Biomedical Research Centre from founders originally obtained from The Jackson Laboratory. CD43<sup>−/−</sup> mice (20) were bred at the Biomedical Research Centre from founders obtained originally from J. Green (Washington University School of Medicine, St. Louis, MO). These mice had been backcrossed on the B6 background for six generations by A. Sperling (University of Chicago, Chicago, IL), and were backcrossed on B6 for two more generations by us before intercrossing heterozygotes to yield CD43<sup>−/−</sup> mice used (21). F7 B6 backcrossed CD43<sup>−/−</sup> mice (6) were provided by J. Marth (Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA). E-selectin<sup>−/−</sup> mice (22) were provided by D. Bullard (University of Alabama, Birmingham, AL) and had been backcrossed on B6 mice in excess of 20 generations. PSGL-1<sup>−/−</sup> and P-selectin<sup>−/−</sup> mice on the B6 background were obtained from The Jackson Laboratory. PSGL-1<sup>−/−</sup>/CD43<sup>−/−</sup> mice were bred in house from the stock strains noted above. The procedures used in this study were reviewed and approved by the University of British Columbia Animal Care Committee.

**Medium and salt solutions**

Cell suspensions were prepared in RPMI 1640 (11875-135; Invitrogen Life Technologies) supplemented with 8% FCS, 5 × 10<sup>−3</sup> M 2-ME, 100 U/ml penicillin, 100 U/ml streptomycin (StemCell Technologies), and 2 mM glutamine (Sigma-Aldrich). HBSS lacking Mg<sup>2+</sup> and Ca<sup>2+</sup> (Hanks’) (cat.-no. 14185-052; Invitrogen Life Technologies) was used in fluorescent tracking dye-labeling procedures. HBSS containing Mg<sup>2+</sup> and Ca<sup>2+</sup> (Hanks’) (cat.-no. H8264; Sigma-Aldrich) was used as indicated. For counting, 100 μl of culture medium, pelleted, washed once in Hanks’, and then suspended in 14 ml of RB and transferred to a 15-ml conical tube (Falcon 2095). Tubes were spun at 1300 rpm for 10 min at room temperature. One 50-ml tube was used for two or three mice depending on the amount of blood. Blood and PBS/EDTA were mixed, and tubes were spun at 1300 rpm for 10 min at room temperature. The supernatant was discarded by aspiration, and the loosened pellet was resuspended in 9 ml of PBS-free nanopure water for 30 s at room temperature to lyse most RBC. One-tenth of added 1% PBN was then filtered through a 5 μm filter. The suspension was then diluted with 40 ml of RB containing 0.2% BSA, 10 mM HEPES, in Hanks’. Tubes were then spun at 1300 rpm for 10 min at room temperature, and the supernatant was discarded. Cells were resuspended in 14 ml of RB and transferred to a 15-ml conical tube (Falcon 2095). Tubes were spun at 1300 rpm for 8 min at room temperature, and the supernatant was discarded. Cells were finally resuspended in 1 ml of RB, an aliquot was labeled with Gr-1 for neutrophil counting. The remaining PBL were labeled with either CFSE or CTO, as described above. For counting, 100 μl of 10-μm latex beads at 10<sup>6</sup>/ml (2–1000; Interfacial Dynamics; Invitrogen Life Technologies) was mixed with an equal volume aliquot of PBN labeled with AlexaFluor 488-conjugated Gr-1 (prepared in house). The bead/cell mixtures were subjected to flow analysis in the presence of 500 ng/ml propidium iodide to determine the ratio of propidium iodide<sup>−/−</sup>/Gr-1<sup>high</sup> PBL/Beads. This ratio was directly proportional to PBN count. Fluorescent tracking dye-labeled PBL were injected i.v. into mice that had just received an i.p. injection of either autolysed thioglycolate (3% w/v dissolved in water; Difco, 243010), or 2% oyster glycogen (G8751; Sigma-Aldrich) dissolved in water (24). Recipient mice received approximately one mouse equivalent of purified donor PBL. Three to four hours later, peritoneal exudates were harvested, stained with allophycocyanin-conjugated Gr-1 (17-5931-82; eBioscience), and analyzed for Gr-1<sup>−/−</sup>CFSE<sup>−</sup> events.

**Western blotting and E-selectin-human Ig (hIg) analysis**

Activated T cells were prepared, as described above, and lysed for immunoprecipitation of either E-SeleL or CD43. Cell lysis and E-selectin-hIg immunoprecipitation were performed, as previously described (13), with some modifications. Briefly, cells were harvested in PBS and lysed at 4°C for 10 min using 1% Triton X-100 in 50 mM Tris-HCl (pH 7.4) containing 150 μM MgCl<sub>2</sub> and 1 mM PMSF for 30 min at 4°C. Three hundred microliters of lysate was preabsorbed with 50 μl of protein A-Sepharose CL-4B (17-0780-01; Pharmacia) for 1 h at 4°C and then for 90 min with either 10 μg of protein A-Sepharose CL-4B (17-0780-01; Pharmacia) for 1 h at 4°C and then for 90 min with either 10 μg
of hlgG (IgG506; Sigma-Aldrich), 10 μg of H18, or 10 μg of E-selectin-hlgG chimera (575-ES; R&D Systems) immobilized on 20 μl of protein-A-Sepharose CL-4B. H18 is a peptide affinity-purified rabbit Ab specific for the cytoplasmic domain of CD43 recognizing all glycoforms of CD43 (25). As a further specificity control for E-selectin-hlg precipitation, 25 mmol EDTA was included during the immunoprecipitation with E-selectin-hlg, where indicated. Protein A-Sepharose was then washed thrice with lysis buffer, and the immunoprecipitate was eluted with 25 μl of gel-loading buffer consisting of 4% lauryl sulfate, 125 mM Tris (pH 6.8), 0.1 mg/ml bromophenol blue, and 20% glycerol. H18 immunoprecipitates were reduced with 5% 2-ME, whereas E-SeIL immunoprecipitates were not; both immunoprecipitates were then heated to 90°C for 5 min and loaded (2 μl of the H18 immunoprecipitate or the entire E-selectin-hlg immunoprecipitate) onto a 7% polyacrylamide gel for electrophoresis and electrot blotting onto polyvinylidene difluoride (PVDF) ECL nitrocellulose (Amersham Biosciences). After transfer, E-selectin-hlg-conjugated Ab and then H18 bound to immunoprecipitated and immobilized CD43 was finally detected with ECL reagent (Amersham Biosciences) and autoradiography with BioMax film (Kodak).

E-SeIL on activated T cells was detected by flow cytometry with the same E-selectin-hlg reagent described above and anti-hlg PE (109-116-dil) and then H18 bound to immunoprecipitated and immobilized CD43 was finally detected with ECL reagent (Amersham Biosciences) and autoradiography with BioMax film (Kodak).

Results
Development of competitive in vivo recruitment model of cutaneous inflammation

Our investigations began with development of a skin recruitment model whereby fluorescent dye-labeled activated T cells expressing high levels of selectin ligand could be injected into a mouse and subsequently visualized at an inflammatory site using a fluorescence dissecting microscope. Efficient recruitment of T cells is currently thought to rely on TCR engagement that acts in concert with chemokine signals to promote firm adhesion and trigger transmigration (26, 27). s.c. Con A administration is reported to stimulate a relatively efficient, P-selectin-, and E-selectin-dependent, s.c. accumulation of T cells (28, 29). Indeed, data implicated a more pronounced role for E-selectin in this recruitment model (29). We therefore chose to test the activity of Con A as an inflammatory agent in vivo and its capacity to attract in vitro activated T cells labeled with fluorescent tracking dyes.

Fluorescently labeled cells were injected i.v. into mice immediately after they had received s.c. injection of 10 μl (10 μg) boluses of Con A. At this dose, a nonulcerating cutaneous inflammation was induced that peaked at day 1 and largely subsided by day 2. The s.c. inflammatory site was examined for accumulations of CFSE- or CTO-labeled dye-positive cells. Preliminary studies showed that dye positive cell accumulations were clearly evident at 18–20 h and followed vascular tracts in the highly vascular region between the s.c. muscle layer and the base of hair follicles; at later time points, infiltrating cells were more abundant, but also more diffusely distributed. Using this experimental procedure, we then assessed whether these cell accumulations reflected a physiologically relevant recruitment process and evaluated the feasibility of competitive recruitment studies in single mice. As shown in Fig. 1A, when two genetically identical donor cell populations were labeled with different fluorescent tracking dyes
and injected into Con A-treated recipients, accumulations of both cell types were clearly present and at apparently equal frequency. To determine whether the use of Con A as an inciting agent altered the requirements typically involved in physiological recruitment, the efficiency of activated C2\(^{-/-}\) T cells was compared with activated B6-derived T cells; C2\(^{-/-}\) T cells lack the ability to generate the branched O-glycan structure on selectin ligands, especially PSGL-1 (6, 30), that are recognized by selectins and necessary for activated T cell recruitment (31). As shown in Fig. 1B, C2\(^{-/-}\) cells accessed s.c. Con A inflammatory sites very poorly relative to B6 T cells. This observation provided evidence that the conditions for accumulation of activated donor T cells relied on effective selectin ligand formation, and thereby replicated physiologically relevant recruitment processes. When CD43\(^{-/-}\) activated T cells were compared with B6 T cells, as shown in Fig. 1C, there was no obvious difference in accumulation efficiency. This result was somewhat unexpected, as recruitment of CD43\(^{-/-}\) neutrophils to inflamed peritoneum had been reported to be defective (15). These results prompted us to quantify the infiltrates and develop a competitive peritoneal recruitment assay for neutrophils.

**Appropriate localization of activated T cell infiltrates in s.c. venules**

To verify that the accumulation of activated T cells at s.c. sites of Con A-induced inflammation reflected a de facto recruitment process, we first needed to demonstrate that donor lymphocytes actually exited the vasculature, did so through postcapillary venules in which lymphocyte emigration occurs most readily (32, 33), and where P (34)- and E-selectins (35) are most abundantly expressed. Vasculature exit was important to establish because transmigration was reportedly impaired in leukocytes lacking CD43 (15). To this end, we developed a simple staining method to discriminate between arteries and veins. We had previously developed a method of i.v. perfusion with CFSE to assess vascular parameters in an Alzheimer disease mouse model (36). In further development of this technique, we found that CFSE perfusion preferentially labels the arterial vascular tree, as shown in Fig. 2, A and B, but labeling is virtually absent on the venous side. This preferential labeling of the arterial vasculature does not reflect biochemical differences between arterial and venous endothelium (as evident in tail vein perfusion studies and high CFSE concentration cardiac perfusion studies; D. Carlow, data not shown), but appears to reflect rapid absorption of CFSE label out of circulation due to the large endothelial cell surface area encountered in the capillary bed. By perfusing with CFSE and then india ink, arterial and venous vascular trees could be readily visualized, as shown in Fig. 2C. When this imaging method was applied to the Con A inflammation model, as exemplified in Fig. 2D, it was evident that cells extravasated, and did so as expected through venules, not arterioles.

**Activated C2\(^{-/-}\) T cells survive in vivo, but exhibit defective recruitment in vivo, whereas activated CD43\(^{-/-}\) T cells recruit normally**

To establish the Con A inflammation procedure as a valid recruitment model, it was essential to show that activated C2\(^{-/-}\) T cells persisted normally in vivo despite their failure to accumulate s.c.
(Fig. 1B). Thus, in vitro viability, in vivo recovery from spleen, and s.c. accumulation of activated donor T cells were assessed in mice that had received B6 and CD43−/− cells or B6 and C2−/− T cells. As shown in Fig. 3, A and B, CD43+/+, CD43−/−, and C2−/− cell viability were maintained in vitro and in vivo (spleen) after tracking dye labeling. When imaging was performed on skin from these mice, infiltrates were counted. Four independent images of infiltrates per mouse were analyzed. Raw count data for red and green cells are summarized in Fig. 3C. C2−/− cells exhibited a clear disadvantage in accessing the s.c. Con A site, whereas CD43−/− T cells and CD43+/+ B6 T cells accessed the Con A site comparably. The data shown in Fig. 3C were further compiled into ratios of green:red cells for each image. Ratios generated for each of the four images from a single mouse were averaged, and the corresponding SD was calculated, as shown in Fig. 3D. In summary, despite the persistence of viable activated C2−/− T cells in vivo, they accessed the Con A-induced s.c. inflammation poorly, confirming that selectin ligand formation was required and validating this recruitment model. Furthermore, CD43−/− T cells exhibited a frequency of recruitment that was not significantly different from CD43+/+ T cells.

**CD43−/− neutrophils recruit normally to inflamed peritoneum**

To assess neutrophil recruitment to inflamed peritoneum, we applied competitive recruitment methods using PBN. Three modifications of the competitive recruitment methodology developed for T cells described above were introduced. First, thioglycolate and oyster glycogen were used as inflammatory stimuli, the latter being the agent formerly used in experiments describing the defect in CD43−/− neutrophil recruitment. Second, to reduce the possibility of dye artifacts affecting recruitment, CFSE alone was used as a tracking dye. Of the fluorescent tracking dyes available, CFSE is relatively intense and is tolerated well. Three low concentrations of CFSE were used that allowed simultaneous flow cytometric enumeration of three independent PBN populations. Third, PBN from peripheral blood were used instead of in vitro activated T cells. Preliminary analysis indicated that despite their low numbers in peripheral blood, native PBN were superior to LPS-elicited or G-CSF-elicited PBN for peritoneal recruitment. PBN can be effectively distinguished by their level of the cell surface marker Gr-1 (37), and this reagent was used to follow PBN specifically. The concentration of viable neutrophils in sample PBN preparations from each mouse strain was determined. PBL were then labeled with distinct CFSE concentrations, combined with other PBL preparations to yield a mixture of PBL containing equivalent PBN numbers from each strain, as shown in Fig. 4A, and then injected i.v. into mice that had just received either thioglycolate or oyster glycogen i.p. Three hours later, PBL and peritoneal exudate cells from the recipient mice were harvested, labeled with Gr-1-APC, and analyzed by flow cytometry for Gr-1high CFSE-labeled cells.

As shown in Fig. 4B, PBL recovered from recipient mice contained essentially equal numbers of B6 and CD43−/− neutrophils, whereas somewhat higher frequencies of C2−/− PBN were present. This was consistent with peripheral blood neutrophilia previously described in C2−/− mice (6), and presumably reflects the failure of these cells to exit the vasculature as a consequence of their inability to synthesize selectin ligand. C2−/− cells accessed inflamed peritoneum very poorly, as shown in Fig. 4C, again as expected (6), but recruitment of CD43−/− cells occurred at a frequency essentially equivalent to CD43+/+ B6 PBN. Data from four independent experiments using both thioglycolate and oyster glycogen inflammatory agents are summarized in Fig. 4D and collectively demonstrated that recruitment of CD43−/− PBN to inflamed peritoneum is not defective.

**FIGURE 3.** C2−/− T cell blasts are maintained in vivo, but fail to recruit in the Con A cutaneous inflammation model, whereas CD43−/− blasts are recruited normally. Four mice received s.c. Con A injections together with tracking dye-labeled i.v. injection of mixtures of B6 and CD43−/− Con A blasts (mixtures 1 and 2) or B6 and C2−/− Con A blasts (mixtures 3 and 4). Dyes were reversed in different mixtures (1 vs 2 and 3 vs 4) to control for dye-related trafficking artifacts. An aliquot of each cell mixture used for injection was placed in culture with IL-2 to monitor dye toxicity. After 18 h, the relative survival of each dye-labeled population was assessed by flow cytometry of in vitro cultured cells (A), by flow cytometry of recipient spleen cell suspensions (B), and by ImageJ software-assisted counting of red vs green cell signals in fluorescence images of the cutaneous inflammation site (four images per mouse; C). The cell-counting data shown in C were compiled as average ratios of CFSE:CTO cell scores determined and shown in D. Recipient mice 1–4 received the following cell-dye combinations: mouse 1 received B6-CFSE plus CD43−/− CTO; mouse 2 received CD43−/− (CFSE) plus B6 (CTO); mouse 3 received B6 (CFSE) plus C2−/− (CTO); and mouse 4 received C2−/− (CFSE) plus B6 (CTO). The data shown are representative of five independent experiments.
and CD43

Assessment of E-selectin and CD43-dependent recruitment using in vivo competition

Recent in vitro studies to elucidate E-SeleL identified the activation-associated glycoform of CD43 as a potential target. Interestingly, CD43 appears to serve selectively as an in vitro ligand for E-selectin, failing to bind P-selectin-Ig chimeras (13, 14). As a cell surface mucin, CD43 is normally highly substituted with O-glycans that can be further modified by C2 upon activation (25) and, at least in human cells, can develop the sialyl Lewis x structure used to generate selectin ligands (38). Our failure to detect a CD43-dependent effect on recruitment in the previous experiments could reflect a redundant function of P- and E-selectins. We therefore sought to analyze CD43-dependent effects on recruitment under conditions in which the influence of P-selectin was eliminated. P-selectin−/− recipients were therefore used with the intent to eliminate recruitment supported by P-selectin and reveal recruitment activity dependent upon E-selectin. The Con A-induced in vivo recruitment of activated T cells appeared to be well suited to address a potential role of CD43 in E-selectin-dependent recruitment because involvement of E-selectin in this system had been previously established (28, 29). To address whether CD43 can contribute to recruitment activity, activated T cells from B6, C2−/−, CD43−/−, and PSGL1−/− mice were generated and paired against B6 control cells in competitive in vivo recruitment assays to cutaneous inflammation in B6, P-selectin−/−, or E-selectin−/− recipients.

Three to four images from each of the recipients shown in Fig. 5 were scored for control B6 and competitor dye-positive cells. These raw count data were expressed as a ratio of competitor: control for each image, and these ratios compiled with ratios from two additional experiments, as shown in Fig. 6. The image data exemplified in Fig. 5 illustrated several points. First, recruitment of C2−/− (Fig. 5B) and PSGL1−/− (Fig. 5D) donor cells was severely compromised, as expected; recruitment of CD43−/− or CD43−/− T cells in B6 recipients (Fig. 5, A and C) was comparable, confirming observations presented in Figs. 1 and 3. Second, overall recruitment of CD43−/− donor cells in PSGL1−/− and in E-selectin−/− recipients (Fig. 5, E–H) was reduced relative to B6 recipients (Fig. 5, A–D), reinforcing the view that both selectins were present and contributing to recruitment in this model. Third, in P-selectin−/− recipients, efficient recruitment maintenance dependence on both C2 and PSGL1 in that cells lacking these genes recruited poorly relative to B6 cells, as shown in Fig. 5E (C2−/−, red; B6 donor cells, green) and Fig. 5G (PSGL1−/−, red; B6 donor cells, green). This result was consistent with the activity of PSGL1 as a known E-SeleL. Finally, B6 CD43−/− and CD43−/−-activated T cells were recruited comparably in P-selectin−/− recipients, indicating that in this in vivo T cell recruitment model, lack of CD43 did not affect recruitment efficiency in P-selectin−/− recipients in which recruitment should depend heavily on E-selectin. In summary, despite compelling data demonstrating that CD43 can bind to CD43 (13, 14), lack of CD43 had no detectable impact on recruitment efficiency in the Con A skin recruitment model described above.

CD43-specific E-SeleL on activated T cells

We anticipated that if CD43 contributed significant E-SeleL function in vivo, it should do so under conditions in which the enzymes forming the selectin ligands on known substrates, such as PSGL1, are active. Previous studies demonstrating E-SeleL interaction with CD43 from cultured murine or human T cells used CD43 purified from anti-TCR-activated T cells that were maintained in IL-2. Furthermore, only the high m.w. (activation-associated) glycoform of

FIGURE 4. Normal recruitment of peripheral blood-derived CD43−/− neutrophils to inflamed peritoneum. PBL from B6, C2−/−, and CD43−/− mice were isolated. Viable Gr-1high PBN were identified and counted, as described in Materials and Methods. PBL from B6, C2−/−, and CD43−/− mice were then labeled with 0.1, 0.03, or 0.01 μm CFSE, respectively, and mixed in proportions that, on the basis of the count data, yielded an input ratio of 1:1:1 ratio of B6, C2−/−, and CD43−/− Gr-1high neutrophils. An aliquot of this input mixture was stained with Alexafluor 488-conjugated Gr-1 and propidium iodide to confirm equivalent input numbers of viable Gr-1high cells from each mouse PBL preparation. As shown in A, gated analysis of the Gr-1high input mixture demonstrated essentially equivalent numbers of the three CFSE-labeled subpopulations. B6 recipient mice received an i.p. injection of either thioglycolate (TG) or oyster glycogen (OG) and then an i.v. injection of the 1:1:1 Gr-1high mixtures of B6, C2−/−, and CD43−/− neutrophils. Three hours later, PBL (B) and peritoneal exudate cells (PEC) (C) were isolated, stained with Gr-1-APC, and analyzed for abundance of each dye-labeled population. Using the recovery of B6 cells (black) as a reference, defined as 1 in each experiment, the ratio of recovered C2−/− (shaded) and CD43−/− (gray) relative to B6 was compiled for each of four experiments, as shown in D. Each triplet histogram summarizes CFSE+ cell yields from one mouse. OG1/PBL refers to analysis of peripheral blood harvested from a mouse 1 h after i.p. injection of oyster glycogen; OG2/PBL corresponds to blood analysis 2 h after injection, etc. IN, Corresponds to input ratios of cells at time of injection for each experiment. Data from four experiments are shown in the four separate clusters of histograms.

CD43-specific E-SeleL on activated T cells

We anticipated that if CD43 contributed significant E-SeleL functionality in vivo, it should do so under conditions in which the enzymes forming the selectin ligands on known substrates, such as PSGL1, are active. Previous studies demonstrating E-SeleL interaction with CD43 from cultured murine or human T cells used CD43 purified from anti-TCR-activated T cells that were maintained in IL-2. Furthermore, only the high m.w. (activation-associated) glycoform of
CD43 exhibited E-selectin-binding activity (13, 14). The T cells we used for the cutaneous recruitment assay were also activated and maintained in IL-2, conditions in which both the activation-associated glycoform of CD43 and P-selectin ligands were heavily expressed (39). However, if activated T cells used in our analyses lacked, for whatever reason, E-SelL on CD43, then conclusions about CD43 function as an E-SelL during in vivo recruitment would not be possible. Therefore, it was necessary to confirm that CD43 molecules on the activated T cells used to assess in vivo recruitment in fact bore de facto E-SelL. To this end, lysates of activated T cells were immunoprecipitated with E-selectin-hIg, and the eluates were subjected to Western blotting with anti-CD43 Ab. As shown in Fig. 7, a high m.w. form of CD43 was effectively immunoprecipitated by E-selectin-hIg, but not by either hIgG or E-selectin-hIg in the presence of EDTA, confirming that CD43-E-SelL was indeed present in activated T cells used in the in vivo recruitment analyses. Use of activated T cells from PSGL-1−/− vs PSGL-1+/+ CD43−/− mice enabled direct resolution of the PSGL-1-dependent, CD43-dependent, and PSGL-1/CD43-independent E-SelL. Cell surface staining with E-selectin-hlg chimera shown in Fig. 7, C and D, demonstrates that the contribution of CD43-E-SelL was minor, but significant ($p < 0.01$) when compared with PSGL-1-dependent E-SelL and other nonPSGL-1/non-CD43 E-SelL. These data are consistent with the previous in vitro observations that CD43-E-SelL can be present on activated T cells (13, 14), and also consistent with our failure to observe CD43-E-SelL-dependent contributions during in vivo recruitment of the activated T cells we have used, presumably due to the low abundance of this entity.

**Discussion**

Two competitive in vivo recruitment assays were used to address the question of whether CD43 participates in the recruitment of effector cells to sites of inflammation. Activated T cells were used to address the question of whether CD43, and in particular CD43-E-SelL interactions, were of importance. PBN were used to assess the relevance of CD43 in peritoneal recruitment. Both assays relied on fluorescent dye tracking of adoptively transferred cells to an inflammatory site, and both assays clearly showed that CD43 and CD43-E-SelL cells were recruited in comparable numbers through postcapillary venules in the Con A-induced cutaneous inflammation model or to peritoneal cavity exposed to either thioglycolate or oyster glycogen in the neutrophil recruitment model. Efficient recruitment in both peritoneal and cutaneous inflammation models was heavily C2 dependent, the most plausible interpretation of this being that selectin ligand formation was necessary. The selectin dependence of recruitment in the Con A cutaneous model was further confirmed with the use of P-selectin−/−, E-selectin−/−, and PSGL-1−/− mice (Fig. 5).

Con A was used to induce s.c. inflammation and has been shown to be a relatively effective recruiting agent in vivo (28). The efficacy of Con A in this regard may stem from its ability to engage the TCR directly (40), thereby providing the TCR signal thought to be necessary for efficient T cell recruitment (26, 27). Con A also
appears to have innate immunity-stimulating activity, as it can induce expression of B7 costimulatory molecules (41, 42) and is also effective at short-term recruitment (4 h) of nonlymphoid cells in peripheral blood to a cutaneous site (D. Carlow, data not shown).

Although we have not assessed in vivo CD43-E-selectin interactions directly, our results do not support the proposition that CD43 can function as an E-Selectin in vivo as in vitro observations would suggest (13, 14). Because rolling is generally a prerequisite for subsequent adhesion and recruitment, it was assumed that if CD43 offered a substantive rolling function via E-selectin, it would translate into improved recruitment particularly in P-selectin−/− recipients. Despite the lack of substantial CD43-dependent activated T cell cutaneous recruitment, CD43-E-Selectin could be demonstrated both biochemically and by cell surface staining with E-selectin-hlg chimera on these T cells (Fig. 7). FACS analysis revealed that CD43-E-Selectin was in low abundance relative to E-Selectin on PSGL-1 and other ill-defined (non-PSGL-1, non-CD43) substrates. The low abundance of CD43-E-Selectin is consistent with our inability to find in vivo relevance for CD43-E-selectin interactions. Notably, preliminary analysis of E-Selectin-hlg staining of PSGL-1−/− vs PSGL-1−/−CD43−/− PBN revealed a similar, very minor, contribution of CD43-E-Selectin (data not shown). It is quite possible that in a different model of inflammation, an in vivo contribution of CD43 as an E-Selectin might be more apparent. However, we have noted both our evidence (re: Fig. 5) and other evidence (28, 29) that E-selectin participates significantly in the Con A-induced cutaneous recruitment model described. Whether alternative T cell activation could promote a more significant expression, and translate into improved recruitment particularly in P-selectin−/− mice, is currently under investigation in our laboratory.

Woodman et al. (15) used IVM to document a >2-fold inhibition in peritoneal recruitment by leukocytes, predominantly neutrophils, in CD43−/− mice relative to CD43+/+ control mice. This outcome was thought to reflect the net consequence of increased adherence to intravascular endothelium and inhibited transmigration by CD43−/− leukocytes. The techniques we have applied do not resolve differences in rolling or transmigration efficiency, but our data demonstrate normal net recruitment efficiency among CD43−/− cells in the assays described. Possible explanations for these conflicting results include the use of noncompetitive experimental formats or CD43−/− mice that were not formally congenic. Onami et al. (18) recently reported that CD8 T cells in CD43−/− mice recruited poorly to LCMV-infected CNS compared with CD8 T cells in CD43+ control mice, and suggested that the CD43−/− recruitment defect was restricted to the CNS sites of inflammation. It would be interesting to test whether the observed difference in recruitment efficiency would persist in a competitive recruitment model of LCMV-specific B6 vs B6.CD43−/− congenic T cells to LCMV-infected CNS.
Whether loss of CD43 significantly affected recruitment; experimental methods to assess recruitment efficiency of activated, CD43 would seem moot. The question of whether CD43 provides an E-SelL on neutrophils in the absence of evidence for CD43-dependent neutrophil recruitment, recruitment efficiency. With clear evidence of C2 dependence and multiparameter capacity. These assays should help to fill a noted gap (1) in experimental methods by providing one option for relatively clear and direct visualization of in vivo T cell recruitment to extralymphatic tissues in a competitive format.

Regarding the neutrophil recruitment assay, methods were applied to isolate neutrophil-rich nucleated cell suspensions from peripheral blood. With minimal manipulations required, including CFSE labeling and adoptive transfer, apparently normal recruitment behavior was preserved in notoriously labile Gr-1high neutrophils. This approach made competitive in vivo recruitment analysis feasible and the conclusion that absence of CD43 did not alter recruitment efficiency. With clear evidence of C2 dependence and absence of evidence for CD43-dependent neutrophil recruitment, the question of whether CD43 provides an E-Sell on neutrophils would seem moot.

In summary, we have developed and presented two experimental methods to assess recruitment efficiency of activated, CD43-deficient, T cells to inflamed skin, and of neutrophils to inflamed peritoneum in a competitive in vivo format. The use of competitive recruitment methods provides leverage in resolving authentic variation in recruitment efficiency. These models were used to evaluate whether loss of CD43 significantly affected recruitment; experiments in both models indicated that loss of CD43 had no impact.

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Disclosures
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